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ACTA CYTOLOGICA

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# The Vaginal Cytology of Gynecologic Endocrinopathies*

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ONE of the rewards of giving a presidential address is the opportunity it provides for presenting material collected over many years in the pursuit of a subject which has become a professional hobby. Indeed, the topic which I have selected is one which I have followed since my medical school days and with which I have had virtually daily contact in the clinic and the laboratory for some 20 years. My purposes are to review briefly some of the historical high lights in hormonal cytology which I have found interesting and significant; to summarize the action of hormones on the vaginal epithelium, and then to present in some detail the vaginal cytology of specific gynecologic endocrinopathies, with particular reference to the usefulness and the limitations of cytology in the differential diagnosis of these conditions.

The earliest use of exfoliative cytology for hormonal evaluation goes back to 1847 when Pouchet, a French physician and zoologist, published a monograph and an atlas[23] on ovulation, in which he not only beautifully demonstrated cyclic changes in the ovary of the human, the rabbit and the pig, but also coincident cyclic changes in the cellular content of the human vaginal secretion. In two plates of drawings showing the microscopic appearance of the unstained vaginal secretion during various phases of the menstrual cycle, Pouchet attempted to demonstrate cyclic variations based on the cellular content. He described the squamous epithelial cells, their increase in number as the mid-cycle is approached, their tendency at this time to be "crowded one upon the other," the subsequent "abundance of the debris of epithelial cells," and degenerative changes and cytolysis indicated by the fact that "almost all the cells are torn into small fragments and completely free nuclei are often found, intact or even in an altered form." He described also the cellular changes during menstruation with the increase in red blood cells, and the presence of "fragments of the preceding decidua." From his various observations Pouchet concluded that (1) The ova are produced and expelled independent of fertilization; (2) The ova are emitted at fixed times which are easily estimated; (3)

* Presidential Address, Eighth Annual Meeting of the Inter-Society Cytology Council, 1960, Chicago, Illinois, U.S.A.

In mammals fertilization takes place only when the passage of the ova into the uterine canal coincides with the presence of the "quickening fluid." He goes on to state that "in order to determine the exact time of fertilization, the intermenstrual period must be carefully studied, whether in woman or in other mammals, for it is during this phase that the egg travels through the uterus or that it stops in the cavity and there undergoes development if it has first met the quickening fluid. In the opposite event, it is immediately expelled."

The suggestion by Pouchet that the vaginal cells may be used as an indicator of ovarian function apparently lay dormant for many years until rediscovered by Stockard and Papanicolaou[36] in 1917 who showed conclusively that cellular changes in the vaginal fluid of the guinea pig could be accurately correlated with corresponding changes in the uterus and ovaries, and especially that the time of ovulation could be readily determined by the vaginal smear pattern. In 1922 Long and Evans[17] described the vaginal smear changes during the estrous cycle of the rat.

At about the same time the studies of Edgar Allen[1] on the cyclic changes in the vagina and the ovary in mice led him to suspect that there is a hormone in the follicles and particularly in the follicular fluid which was responsible for the events of estrus and in subsequent studies with Doisy[3] was able to show that the vaginal smear technic could be used as a specific test for the follicular hormone.

In the next several years similar vaginal cytologic studies were made in other animal species, including the observations of Corner[7] and of Allen[2] in the rhesus monkey indicating that there was to some degree cyclic changes in the vaginal secretion in this species; however, these workers were not able to accurately determine the presence or absence of ovulation by the criteria which they used, which were based mainly on the relative numbers of leukocytes and desquamated epithelial cells rather than on the morphologic characteristics of the latter.

During the nineteen-twenties a number of papers appeared recording observations on the vaginal histology and vaginal cytology in women which made it evident that the cyclic changes in the human vagina were more difficult to evaluate than the clear-cut patterns found in rodents. (For a critical review of these articles the reader is referred to the Papanicolaou monograph.[21])

The real break-through for hormonal cytology in the human came in 1933 with the publication by Papanicolaou of a classic monograph entitled "The Sexual Cycle in the Human Female" which appeared as a supplement to *The American Journal of Anatomy*.[21] Clinical hormonal cytology as we know it today dates from this publication. Here we find a description of the aspiration method of collecting vaginal fluid with the pipette and rubber bulb, the fixation method with ether and alcohol, a staining method using hematoxylin, eosin and water-blue, and a detailed description with excellent drawings of the various types of cells found in the normal vagina under different physiologic circumstances and a description of the cyclic changes during the normal menstrual cycle. At this time Papanicolaou divided the female cycle into four phases and one stage: (a) the menstrual phase (first to seventh days), (b) the copulative phase (eighth to 12th days), (c) the ovulative stage (12th to 13th days in the most typical cases), (d) the proliferative phase (13th to 17th day), (e) the premenstrual phase (17th day up to the next menstruation). In this monograph Papanicolaou also commented on the vaginal smear changes in pregnancy which he had actually previously reported in 1925,[20] and also the postpartum type of smear.

**Effect of Hormones on the Human Vagina**

To properly interpret the vaginal cytol-

ogy smear in various endocrinopathies it is necessary first to consider the effect of various hormones on the vaginal epithelium, and also the influence of other factors which may alter the response of the vaginal epithelium to endogenous hormones.

Although the effect of estrogens on the vaginal epithelium is the most obvious and the best known of the hormonal influences on vaginal histology and cytology, it soon became apparent to the early workers in this field that progesterone had a modifying influence on the estrogenic effect on the vaginal epithelium, and it was subsequently shown that androgens also exerted a modifying influence. Further investigations showed that many steroid hormones of gonadal, adrenal, and placental origin may influence the total cytologic picture thus often making the cytologic interpretation of the hormonal status difficult in the presence of various hormonal aberrations.

**Estrogens:** The effect of estrogens on the vaginal epithelium is too well known to this audience to require detailed description. Suffice it to say, that of all the hormones, estrogens have the most marked effect on inducing proliferation of the vaginal epithelium and, in addition, are responsible for the maturation of the cells of the vaginal epithelium as indicated by various morphologic and cytochemical characteristics.

The effect of estrogens on the human vagina on the basis of these criteria can be quantitated with reasonable accuracy in the castrate woman, or in normal women during the estrogenic phase of their cycle, but it is more difficult to evaluate estrogenic action in the presence of other steroid hormones, or certain other factors which may imitate, modify, obscure or interfere with the effect of estrogens on the vaginal epithelium. In particular it is well known that trichomoniasis and other infections of the vagina may make it impossible to accurately read the estrogenic effect. Vitamin deficiencies, metabolic disturbances, and genetic factors may alter the target organ sensitivity.

There has been considerable controversy regarding a suitable nomenclature or classification for quantitative estimation of "estrogen effect" on vaginal smears. Various schemes of classification have been described including a description of varying degrees of atrophy and proliferation, maturation indices, and eosinophilic and karyopyknotic indices, as well as varying descriptive terms based on resemblance to the findings during a normal menstrual cycle or in various physiologic periods of life.[9, 11, 20, 27, 33, 40] It must also be borne in mind that although the vaginal smear is very sensitive to changes in the concentration of estrogens found during the normal cycle or in postmenopausal women, it cannot accurately reflect the influence of higher titers of estrogens except to indicate a "marked estrogen effect."

It behooves the clinician to become aware of the criteria employed in his particular laboratory and furthermore to interpret the results in the light of the over-all clinical findings including particularly the age of the patient, the time of the cycle, the type of menstrual pattern, the presence of infection in the genital tract, recent hormonal therapy, extra-gonadal endocrinopathies, and metabolic disturbances.

**Progesterone:** The effect of progesterone on the human vaginal epithelium is dependent on its immediately previous degree of atrophy or proliferation and whether or not estrogens (or other steroids) are acting upon the epithelium at the same time. Thus, for example, the effect of progesterone acting alone on the atrophic vagina is minimal producing at most only a small growth stimulating effect; on the other hand, on the estrogen-primed epithelium progesterone causes increased desquamation and "regressive" changes on the superficial proliferated epithelium evidenced by

curling of the edges, folding, clustering and degenerative changes. In a woman with a normal corpus luteum, these changes occur in a more or less "regular" pattern over a 14-day period, but when there are fluctuations in the amount of estrogens or progesterone secreted the pattern is bizarre and difficult of interpretation. Furthermore, the effects of progesterone are not at all specific and may be imitated by a drop in the pre-existing estrogen level or by an increase in androgens. For these various reasons it is apparent that although the cytologist can, with considerable accuracy, indicate the onset of luteal function in a normal cycle and the relative normality of the corpus luteum phase when provided with daily vaginal smears, progesteronic function cannot be ascertained with confidence from random smears. On the other hand, under known experimental condition it is possible to evaluate the effect of administered progesterone on a previously well proliferated vaginal epithelium. This has been nicely demonstrated by Wied, *et al.*,[42] employing criteria of clustering and folding as indices.

It should also be mentioned that some of the new synthetic progestational compounds such as Norethynodrel and Norethenidrone exert a multihormonal effect on the vaginal epithelium. This is a reflection of the fact that these hormones have estrogenic, androgenic and progesteronic actions.

**Androgens:** As in the case of progesterone the effect of androgens on the vaginal epithelium is also influenced by numerous factors. The amount of androgen normally present, represented by a urinary 17-ketosteroid excretion of 5 to 15 mg. per 24 hours, has little or no demonstrable effect on the vaginal epithelium. This is indicated by the normal cyclic smears which one can observe in some adrenalectomized young women supported with small doses of cortisone alone. If larger amounts of androgen are present as a result of excessive endogenous production by the adrenal or ovary, as occurs in virilizing syndromes, or as a result of exogenous administration, the effect will be the resultant of numerous forces including (1) the direct effect of the androgen on the vagina (2) the inhibition of pituitary-ovarian function by the androgen and (3) the conversion of a small amount of the androgen to estrogen metabolites. Furthermore the direct effect of the androgen on the vaginal epithelium will be markedly influenced by the pre-existing relative degree of proliferation or atrophy of the vaginal epithelium.[5, 6, 10, 30, 39] It is now generally agreed that androgens in sufficient dosage acting upon an atrophic epithelium will produce some degree of proliferation of the parabasal cells and may even cause some growth of intermediate cells but not usually of cells of more mature type. On an atrophic epithelium, therefore, the effect of androgen is one of intermediate proliferation. On the other hand, however, if the vaginal epithelium is already well proliferated, the effect of androgen is in the direction of atrophy. It is not surprising, therefore, that in virilizing syndromes the cytologic picture may be quite varied and sometimes bizarre.

A number of the European workers particularly Pundel[24, 25] and Gompel,[12] are of the opinion that the presence of androgen in greater than physiologic amounts may be detected by the presence of a particular cell type which they refer to as the "androgenic cell," or a particular pattern referred to as the "androgenic smear."

Pundel[25] describes the androgenic cell type as follows: "The vaginal cells under pure androgenic stimulation are exclusively of the parabasal or intermediate type, with a high glycogen content in the cytoplasm producing a pale basophilic staining of the cytoplasm. The nucleus remains large and stains pale with chromatin disposed in very minute granules. Determination of the

D.N.A. content of these nuclei under microphotometry seems to indicate a relatively lower D.N.A. content compared with that of nuclei from the same cell type under pure estrogenic stimulation." Pundel also notes that cytologic characteristics of the androgenic cells appear vividly after staining with the Shorr S30 hematoxolyn technic,[28] whereas the Papanicolaou technic occasionally shows a lighter eosinophilic reaction of the cytoplasm and a more compact nucleus. It is puzzling that he has been able to produce this androgenic cell type only with pure testosterone, testosterone proprionate or testosterone acetate, while other hormones with adrogenic activity frequently produce a more or less multi-hormonal effect. With regard to its significance he states that the androgenic smear indicates only that there exists a predominance of androgens in the patient.

Gompel in the same symposium in *Acta Cytologica*,[12] states that a distinction should be clearly made between the "androgenic cell type" and the "androgenic smear type." Other elements such as vaginal *p*H, the bacterial flora, and the absence of eosinophilic pyknoptic cells and leukocytes are also characteristic of androgenic stimulation. He indicates however that the absence of androgenic cells does not mean that there is no male hormone activity and therefore the presence of that cell type cannot be used as a quantitative test of androgenic activity.

From our own observations[30] I have never been able to convince myself that the so-called androgenic cell is specific for increased androgenic activity, particularly since similar type cells may be found in some patients with varying degrees of ovarian hypofunction and normal 17-ketosteroids.

**Adrenal Steroids:** In usual therapeutic doses desoxycorticosterone exerts little if any demonstrable effect on the vaginal epithelium. In higher dosages Montalvo[18] reported an effect similar to that obtained with progesterone. Cortisone and related corticosteroids also exert a progesterone-like action in high dosage[11] but again in usual therapeutic amounts we have observed but little effect.[31] In contrast, when cortisone is given to patients with virilizing adrenal hyperplasia, a striking proliferative effect is obtained as a result of the androgen inhibition and subsequent release of ovarian function.

**Other Hormones and Vitamins:** Comparatively little is known of the direct influence of various other hormones on the vaginal epithelium such as growth hormone or thyroid hormone, although there are observations to suggest that these at least influence the response of the target vaginal epithelium to the steroid hormones.[27]

The influence of vitamins also require consideration in view of the fact that vitamin deficiency states or altered vitamin metabolism may occur in association with some endocrinopathies. Certain vitamins, such as A and D and E, apparently exert a direct influence on epithelial structures, while the lack of others may influence or modify the action of the steroid hormones on the vaginal epithelium.[27, 31]

**Vaginal Smears for Hormonal Diagnosis**

Although there is an abundant amount of material in the literature concerning the effects of various hormones on the vaginal cytology smear, there is a relative dearth of descriptions of the hormonal cytology in specific endocrinopathies in spite of the fact that cytology smears are now used almost routinely as part of the endocrine work-up in women with gynecologic dysfunctions and other endocrine problems. There are but few systematic descriptions of the hormonal cytology in various endocrinopathies[9, 27] and it is especially for this reason that I am attempting to draw together my own observations in this presentation. I do this particularly, also, because in the practice of endocrinology I have

found a vaginal smear to be of considerable value in differential diagnosis, particularly in ruling in and ruling out certain possibilities, but at the same time I should like also to indicate those conditions and circumstances in which the vaginal smear is of limited usefulness.

Although it is hardly necessary to discuss details of technic with this audience, I think that certain general statements with regard to methodology for hormonal cytology are in order.

**Preparation of Smears:** When smears are specifically taken to determine hormonal function there can be little doubt that the most satisfactory site is from the lateral wall of the middle third of the vagina, in order to obtain cells which are relatively fresh and to avoid the contamination of the cervical secretions. To prevent drying we use either non-absorbent cotton on an applicator stick, or the ordinary cotton-tipped applicator dipped in saline. In addition to the usual permanent smear we have also been in the habit of preparing a wet smear for rapid use[29] which can be read while the patient is still on the examining table and which very often provides helpful information necessary at the moment. For this purpose the secretion collected on a saline moistened cotton-tipped applicator is dropped at once into a test tube containing 2 cc. of saline. Three drops of the stain (Light Green, 5 per cent aqueous solution, 83 parts and Eosin Y, 2 per cent aqueous solution, 17 parts) are added, gently mixed with the applicator and then after a minute or two a drop transferred by means of the applicator to the glass slip, covered, and examined at once under the microscope. We also make it a habit of examining a drop of the unstained material (before the dye is added) for the presence of trichomonads, fungi, and leukocytes, since these may indicate the presence of factors which may alter the validity of the vaginal smear reading.

Since, in almost all instances, such smears are collected with the speculum in place, it is valuable from a hormonal standpoint to also check the cervical mucus at the same time. If no cervical mucus is present a drop is aspirated with a bacteriological pipette and tested for Spinnbarkeit, Fern reaction, and cellularity.

For the staining of the fixed smear for hormonal purposes, it is helpful to have good cytoplasmic staining to differentiate the cyanophilic and eosinophilic cells distinctly and this should be borne in mind in adjusting the staining time if the usual Papanicolaou stains are used. In this respect the Shorr stain[35] is very helpful and has the advantage of giving deeper cytoplasmic staining, but there is of course loss of nuclear detail unless hematoxylin staining is added.[28] The original eosin and water blue stain[21] used by Dr. Papanicolaou also affords very pretty pictures of cytoplasmic differentiation.

In those cases in which for one reason or another vaginal smears cannot be ob-

TABLE 1. *Primary Amenorrhea*

| Etiology | Hormonal cytology smear | Nuclear sex |
|---|---|---|
| 1. Gonadal dysgenesis | Markedly atrophic | "Negative" in 80% |
| 2. Ovarian eunuchoidism | Markedly atrophic | "Positive" |
| 3. Pituitary hypogonadism | Atrophic to mod. proliferative | "Positive" |
| 4. Uterine defect | Proliferative, cycling | "Positive" |
| 5. "Feminizing testis" syndrome | Proliferative, slight to mod. | "Negative" |
| 6. Congenital adrenal hyperplasia | Atrophic or intermediate proliferation | "Positive" |

tained smears prepared from urinary sediment may be used, since it is well known that the urethral epithelium also adequately reflects the hormonal cytology.[9]

### Clinical Applications

**Primary Amenorrhea:** In a high proportion of instances primary amenorrhea results from a congenital defect of the gonads or of the reproductive tract, and it is for this reason that cytology smears are of particular value and significance in establishing the diagnosis. In Table 1 are listed the causes of primary amenorrhea and the cytology smear findings in these conditions.

*Gonadal Dysgenesis:* Of particular interest are the cases now listed under gonadal dysgenesis, which were formerly classified under a variety of terms such as Turner's syndrome, ovarian agenesis, ovarian dwarfism, ovarian aplasia, and others. These patients are usually characterized clinically by evidences of hypogonadism including lack of sexual maturation and primary amenorrhea, usually shortness of stature, and sometimes by other congenital defects including webbing of the neck, pigmented moles, telangiectasia, polydactalism, coarctation of the aorta, osteoporosis, and numerous others. This condition is caused by a genetic defect in which there has been failure of the gametes to either migrate into the urogenital ridge or to survive, so that the gonad fails to develop as such and is represented only by a band of fibrous tissue containing stroma and occasionally a few tubular remnants or an occasional follicle. Such a gonad, of course, produces no estrogen and, as might be expected, the vaginal cytology smear is of a markedly atrophic type showing only deep parabasal type cells. It was suspected for some time that many of these cases although appearing as phenotypic females might actually be males chromosomally, and in whom, because of lack of fetal androgen production, the mullerian system went on to develop and to form the female type of genital tract. With the discovery of technics for the determination of chromosomal sex this was indeed confirmed and to make matters more interesting it now appears that about 80 per cent of the cases of gonadal dysgenesis are actually males. It is for this reason that it is very important to do chromosomal sex determination on all patients with primary amenorrhea. This should be done even though the patient may not show some of the other stigmata of Turner's syndrome such as shortness of stature, or various congenital anomalies, since this syndrome can occur occasionally in patients of normal stature and not infrequently in the absence of other congenital defects. Indeed there is now reason to believe that this syndrome is responsible for a majority of cases of primary amenorrhea.

It is of interest to speculate whether the vaginal smears in patients with gonadal dysgenesis show any differences so far as estrogen effect or estrogen response is concerned in the chromosomal females as compared to the chromosomal males. Except for the presence or absence of the nuclear satellite, in most instances we have found no such differences. There is, however, a very occasional patient with gonadal dysgenesis in whom the smear is not completely atrophic. This suggests that there may be some estrogen secretion by epithelial remnants in the gonads or by the adrenals.

It is to be emphasized at this point that the vaginal smear is generally as satisfactory as the buccal smear for this determination of nuclear sex, and indeed the same Papanicolaou stained smears used for hormonal cytology can be used for this purpose, counting all cases as "positive" (female) which show a satellite count of 20 per cent or more. In doubtful cases thionin[16] or Guard[13] stained preparations are recommended.

The nuclear sex determination has been of particular value in the early diagnosis of Turner's syndrome in children who are

failing to grow properly. Since the chromosomal sex is "negative" (male) in 80 per cent of the cases, the majority can now be detected even in early childhood if the diagnosis is suspected on the basis of shortness of stature or the appearance of some genetic defect. An additional laboratory procedure of value, particularly in the cases with chromatin positive patterns is the demonstration of high gonadotropins in the urine, which may be demonstrable by the age of six or sometimes earlier.

*Ovarian Eunuchoidism:* Another type of primary amenorrhea which is also associated, with a markedly atrophic vaginal smear is that seen in the syndrome of ovarian eunuchoidism. In this condition the ovaries have been irreparably damaged during childhood by such causes as mumps oophoritis, or other virus or bacterial infections, or injudicious x-ray exposure. In these cases the individual is also hypogonad and has primary amenorrhea but in addition the skeletal proportions are eunuchoid and the individual is quite tall. The gonadotropins in this condition are also quite high. The nuclear sex in these cases however is invariably "positive" (female type).

*Pituitary Hypogonadism:* In primary amenorrhea associated with hypogonadism of pituitary origin the nuclear sex is also always satellite positive, but the clinical picture and the hormonal cytology are subject to considerable variations. In most instances the individual may be dwarfed, because of a concomitant deficiency of growth hormone, but occasionally may be tall and eunuchoid (pituitary eunuchoidism). Although the vaginal smear may, in some instances, be markedly atrophic, not infrequently some degree of proliferation is present, as indicated by the presence of intermediate cells, and occasionally some superficial cells.

*Primary Absence of Uterus:* In patients with a simple congenital absence of the uterus but with normal gonads the vaginal smear will show not only a good proliferative pattern but also repeated daily smears will show normal cycling. In such instances the nuclear sex pattern is of the satellite positive type. An interesting variation is the patient with primary amenorrhea who has a markedly hypoplastic uterus which is irresponsive to ovarian hormones even when the latter are given exogenously in large doses. In these patients the vaginal smear is of the normal proliferative type and also shows cycling, indicating that the uterine irresponsiveness has not involved the lower portion of the mullerian tract.

In the patient who is born with a congenital absence of the vagina as well as the uterus a dimple of mucous membrane is usually present at the site of the expected vaginal orifice. By long-term pressure with graduated dilators this dimple may eventually be invaginated and dilated to the point where it forms a functional vagina. It is of interest that the lining vaginal epithelium so formed generally shows a good response to the ovarian hormones as indicated by normal cyclic vaginal smears.

*Feminizing Testicular Syndrome:* This rare syndrome is a type of pseudohermaphroditism seen in an apparently normal young female who generally presents herself with the chief complaint of primary amenorrhea. On examination adequate breast tissue is present, pubic hair is scant, the external genitalia are normal, the vagina may be normal or short, but there is a congenital absence of the uterus. The gonads in some cases may descend and present themselves in an inguinal hernial sac. Histologic examination reveals these to be testes which have apparently in some way been affected during intra-uterine fetal life. Spermatogenesis is absent but the lining sertoli cells are well preserved and are probably the site of the obvious estrogen secretion. The vaginal smear shows slight to moderate proliferation. Of the five cases which we have seen, the smears in three showed cyano-

philic superficial cells predominating with the presence of some intermediate cells, while in two there were chiefly superficial cells with an eosinophilic index of 20 to 40 per cent. Urinary hormone assays showed normal 17-ketosteroids and the estrogen bioassay showed estrogens present in amounts within the normal range for an adult female. Following removal of the gonads the smear became moderately atrophic and the gonadotropins elevated. In two cases of testicular feminization observed by Berger,[4] the vaginal smears showed an "increased estrogenic effect" in spite of a low biologically assayed estrogenic value, and diminished excretion of phenolic steroids and increased 17-ketosteroids in the 24-hour urine. He notes that Schaumkell and Goldberg made similar observations.

The nuclear sex in these cases is satellite negative.[8] Actual chromosomal counts indicate the presence of an XY pattern.[14]

*Congenital Adrenal Hyperplasia:* To complete the list of genetic defects which may be responsible for primary amenorrhea we add the condition of congenital adrenal hyperplasia. This is a masculinizing syndrome due to an inborn error of metabolism in which, in most instances, there is a defect in the enzyme system dealing with the conversion of hydroxy-progesterone to cortisone resulting in the piling up of 17-ketosteroids and pregnantriol. As a consequence the developing female fetus is subjected to increased amounts of androgen resulting in enlargement of the clitoris to form a phallus and often the production of a urogenital sinus. Rarely fusion of the labio-scrotal folds may occur. In the latter cases the assigning of sex at birth may be difficult. In such instances the nuclear sex determination is very helpful since it is always satellite positive in this syndrome.

In the typical case the vagina is not easily accessible since it lies within the urogenital sinus. With the aid of a probe wrapped with cotton and moistened with saline, vaginal smears can generally be obtained, or smears can be prepared from urinary sediment. The vaginal epithelium in the untreated case varies considerably. In some instances it is completely atrophic, in others an intermediate type of proliferation is present with the presence of cells from all three layers. This perhaps might be expected in view of the fact that varying amounts of 17-ketosteroids of varying androgenic activity are present in these patients. In some instances ovarian function is not completely suppressed and follicular cystosis of the ovaries may occur. Following cortisone therapy a normal proliferative cyclic type of smear is obtained, since the administration of cortisone depresses the overactive adrenal thus releasing cyclic ovarian function.

**Polycystic Disease of the Ovaries:** Patients with polycystic disease of the ovaries are of particular interest from the standpoint of their gynecologic-endocrine status. The clinical and hormonal findings may vary considerably. Although it is difficult to subdivide these accurately, for purposes of this discussion three main groups may be recognized.[19, 21]

(1) *Patients with Follicular Cystosis and Hyperestrogenism:* Women in this category usually present no endocrine manifestations except for a menstrual disorder characterized by marked menstrual irregularity, often with episodes of oligomenorrhea followed by severe hypermenorrhea. The ovaries show multiple follicle cysts sometimes with hyperplasia of the theca or stroma. The capsule may or may be not markedly thickened. This condition is thought by some investigators to result from an imbalance of the gonadotrophic hormones. The vaginal cytology smear is generally highly proliferated and except for episodes of desquamation shows no cyclic changes. The cervical mucus is generally copious, thin and watery.

(2) *The Stein-Leventhal Syndrome:*

TABLE 2. *Syndromes with Virilization or Hirsutism*

| Etiology | Smear type |
|---|---|
| 1. Simple (genetic) Hirsutism | Normal, cycling |
| 2. Adrenogenital syndrome — Hyperplasia, Tumor | In all of these the smear pattern varies |
| 3. Cushing's syndrome — Hyperplasia, Tumor, Pituitary adenoma | A. Atrophic |
| 4. Masculinizing ovarian tumors | B. Intermediate proliferation |
| 5. Stein Leventhal syndrome | C. Atypical ("multihormonal") smears |
| | D. Occasionally highly proliferative smears |

From a clinical, hormonal, and pathologic standpoint this condition is a poorly defined one. Perhaps the only constant features are polycystic disease of the ovaries, thickening of the capsule, and menstrual irregularity. The so-called "typical Stein-Leventhal" patient is a woman in her early reproductive years, who developed progressive menstrual irregularity in her late teens, now has secondary amenorrhea or marked oligomenorrhea, anovulatory infertility, mild to moderate hirsutism, but usually no other evidences of frank masculinization, and may be moderately obese. The ovaries are usually enlarged, pearly-gray in color and have a smooth thickened capsule, with numerous small follicular cysts of varying size which are evident in the cortex on the cut section of the ovaries. Not infrequently there is hyperplasia of the stroma and the theca interna surrounding the follicle cyst. Hormonal assays generally show a low or normal FSH, normal or slightly elevated 17-ketosteroids, absent pregnandiol, and small to moderate amounts of estrogens.

Vaginal smears show a slight to moderate degree of proliferation in the majority of cases. Many exceptions to the typical findings listed above may be encountered (Table 2). In some instances the patient may show a well proliferated type of smear with a fairly high eosinophilic and karyopyknotic index. These patients will sometimes have hypermenorrhea and polymenorrhea other than amenorrhea. In other instances smears containing chiefly intermediate and parabasal cells may be present and in some of these cases the 17-ketosteroids may be distinctly elevated. The latter cases probably represent patients with mild adrenal cortical hyperfunction who have developed a secondary polycystic disease of the ovaries. Indeed polycystic disease of the ovaries, quite indistinguishable from the so-called Stein-Leventhal ovaries, may also occur in patients with congenital adrenal hyperplasia and in patients with adrenal cortical tumors.

(3) *Frank Virilization:* Women with bilaterally enlarged ovaries with more marked masculinization than is generally noted in the usual case of Stein-Leventhal syndrome may show extensive luteinization of the ovaries or hyperplasia of the hilar cells. The 17-ketosteroids are usually normal, but in some instances may become elevated, particularly during pregnancy. Estrogen secretion also varies considerably and some cases have been reported with evidences of hyperestrogenism. Vaginal smear data on such cases are rare, but in the instances which have come under our attention have varied from an atrophic pattern to persistent cornification.

**Other Masculinizing Syndromes:** In this category are listed patients with acquired hyperfunction of the adrenal cortex due to hyperplasia or tumor, giving rise to an adrenogenital syndrome or Cushing's syndrome, and also patients with masculinizing tumors of the ovary (Table 2).

*Genetic Hirsutism:* It should be pointed out first that the cytology smear is of value in separating the above conditions from patients with simple hirsutism of genetic origin. The latter show normal cyclic smears,[26, 37] and also generally have a normal menstrual pattern, unless there is some

other associated factor inhibiting ovarian function. Furthermore in genetic hirsutism the urinary 17-ketosteroid levels are normal.[22]

*Adrenogenital Syndrome:* In patients with virilism resulting from adrenal cortical hyperfunction or a masculinizing tumor of the ovary the vaginal smear generally shows an atrophic pattern or some type of intermediate proliferation. In some instances the smear may be rather bizarre showing parabasal cells, intermediate cells, precornified cells, and occasionally even some cornified cells. The finding of some rather well differentiated superficial cells in a basically atrophic type of smear arouses suspicion of combined estrogen and androgen action, but it should be emphasized that there is no one clear-cut cytological pattern for any one of the endocrine causes of hirsutism. Pundel[26] who comes to the same conclusion mentions some patients with very high 17-ketosteroid levels (up to 300 milligrams per 24 hours) in which the vaginal cytology was purely of an estrogenic type and in whom the endometrium exhibited a typical glandular hyperplasia of the Swiss-cheese type. He concluded that the vaginal smear is not a sensitive test that enables a separate estimation of the androgenic activity in the female, but rather reflects the summation of the different hormonal actions. I have personally never been convinced that there is a cell type which is specific for androgenic activity or even an androgenic type of smear which is specific for masculinizing syndromes. I do believe that these syndromes can be suspected when one finds an atypical smear predominantly of an atrophic type but with some proliferative elements, however in the majority of cases one finds either frankly atrophic smears or smears showing intermediate types of proliferation which, as Wied[41] also points out, are nonspecific.

*Cushing's Syndrome:* In Cushing's syndrome the vaginal smear is also of limited value. In our experience the smears may vary from a completely atrophic type to those showing a normal proliferative type of smear and some even with cycling. The latter occurred in one of our patients who became pregnant while she had this condition. Berger[4] refers to cases with "absolute atrophia" in the vaginal smears. This marked variation in Cushing's disease might be expected in view of the great variation in the androgen and estrogen pattern in these patients. Some may have 17-ketosteroids which are actually in the normal level while others may have markedly elevated androgens. There does not appear to be any specific relationship of the vaginal smear to the height of the corticosteroid level.

TABLE 3. *Psychogenic Amenorrhea*

| Etiology | Smear type |
|---|---|
| 1. Pituitary inhibition | |
| a) FSH & LH | Atrophic to intermediate proliferation |
| b) LH | Intermediate proliferation |
| 2. Ovarian inhibition | Markedly atrophic |
| 3. Persistent estrus | Proliferative. High K.I. & E.I. |
| 4. Pseudocyesis | Progestational types |

We have not found the cytology smears to be of value in differentiating cases of either the adreno-genital syndrome or Cushing's syndrome due to hyperplasia contrasted to those due to tumors.

*Masculinizing Ovarian Tumors:* Very few observations have been reported on patients with masculinizing tumors of the ovary. In two patients with arrhenoblastoma we found markedly atrophic smears, while in a child with an adrenal-like ovarian tumor and a young woman with a lipoid cell tumor the smears showed atypical smears containing primarily parabasal and intermediate cells but also occasional superficial cells.[34]

**Psychogenic Amenorrhea:** It is now well recognized that nervous and emotional dis-

TABLE 4. *Amenorrhea with Galactorrhea*

| Etiology | Smear type |
|---|---|
| 1. Normal lactation | "Post-partum" type |
| 2. Chiari-Frommel syndrome | Markedly atrophic |
| 3. Chromophobe adenoma of pituitary | Atrophic to intermediate proliferation |

turbances are a common cause for menstrual dysfunction, particularly secondary amenorrhea, in young women. A number of physiologic mechanisms, whereby such factors may disturb the normal cycle have been suggested (Table 3).

In the majority of cases the disturbance operates on a hypothalamic level affecting the secretion or release of the pituitary gonadotrophic hormone. In some instances, however, the evidence suggests that the ovary may be inhibited directly, perhaps by way of its sympathetic nerve supply. The more common hormonal pattern seen in patients with psychogenic amenorrhea and the corresponding findings on the vaginal smear are shown in the accompanying table.

In the first pattern we note that we have inhibition of both follicle stimulating and luteinizing gonadotrophic hormones, resulting in varying degrees of ovarian deficiency, so that the smear may vary from a markedly atrophic type to one showing intermediate proliferation, depending upon the degree of ovarian inhibition. The atropic pattern is seen particularly in women who have had an acute emotional upset which was followed promptly by secondary amenorrhea, or in patients with longstanding severe psychoneurosis. Women with the more severe examples of this syndrome may also show relative target organ insensitivity to estrogen and progesterone, so that there may be marked difficulty even in inducing menstrual bleeding with large amounts of these hormones. The uterus in these cases proves to be more insensitive than the vagina, since frequently bleeding will fail to occur, even though the vaginal smear has shown an adequate response.

Another type of secondary amenorrhea operating by way of the hypothalamus and originally described by Klinefelter and his associates[15] as "hypothalamic amenorrhea" apparently is characterized by inhibition of LH alone with normal or near normal titers of FSH. The vaginal smear type here is generally one of intermediate proliferation, rather than marked atrophy. This is a common pattern seen in young women under various stress situations.

In occasional instances, acute emotional trauma may apparently cause inhibition of the ovary directly, resulting in high FSH level and a markedly atrophic vaginal smear type. Such patients also generally complain of menopausal symptoms, including hot flashes and other vasomotor disturbances.

Still another pattern of hypothalamic amenorrhea is that of a persistent estrus smear with a good proliferative effect on the smear and a high eosinophilic and karyopycnotic index, which, however, does not show much fluctuation over a long period of time. In such instances there is apparently a constant production of FSH, but absence of LH. Indeed, such patients can often be thrown into ovulation by the administration of chorionic gonadotrophin, resulting in a sharp rise in their basal temperature chart and the occurrence of a regressive type of vaginal smear.

*Pseudocyesis:* An interesting type of hyperhormonal amenorrhea resulting from psychogenic factors is seen in the pseudocyesis syndrome. In this condition, a woman who strongly desires pregnancy, but who, for various reasons, may not be able to become pregnant, develops a conversion hysteria causing the appearance of many symptoms suggesting pregnancy. In part, this is induced by a hypothalamic dysfunction causing persistence of LH and LTH, and therefore continued stimulation

of the corpus luteum. The latter continues to secrete estrogen and progesterone over prolonged periods of time, thus simulating the hormonal picture of early pregnancy, resulting in a hyperhormonal type of amenorrhea and stimulation of the breasts. The vaginal smear here may be of the frankly progestational type, or may show an atypical regressive picture.

**Amenorrhea with Galactorrhea:** The secretion of milk or a milky discharge from the breasts with amenorrhea may occur not only during the normal postpartum period and during the subsequent months of nursing, but may also occur in association with certain endocrinopathies such as the Chiari-Frommel syndrome, chromophobe adenomas of the pituitary, or sometimes in association with other intracranial lesions impinging upon the pituitary or hypothalamus (Table 4).

The hormonal pattern during normal lactation is well-known. It is characterized by persistence of secretions of lactogenic hormone but inhibition of follicle-stimulating hormone, so that ovarian function is minimal. The vaginal smear in such instances is quite characteristic and is referred to as "postpartum type."

*Chiari-Frommel Syndrome:* In the Chiari-Frommel syndrome the endocrine mechanism is somewhat similar to that seen in normal lactation, and indeed begins in the postpartum period, but is characterized by prolonged persistence of the secondary amenorrhea over many years, together with the persistence of a milky discharge. Another well-known characteristic of this syndrome is the hyperinvolution of the uterus and marked atrophic changes in the vagina. As a result, the vaginal cytology smear in the true Chiari-Frommel syndrome is one of marked atrophy. This is often of help in differentiating the true Chiari-Frommel syndrome from other types of amenorrhea with galactorrhea, since the latter do not often show the marked degree of vaginal atrophy seen in the Chiari-Frommel syndrome.

TABLE 5. *Feminizing Precocious Puberty*

| Etiology | Smear type |
|---|---|
| 1. TRUE | |
| a) Constitutional | Varies |
| b) Intracranial lesions | Proliferative |
| 2. PSEUDO | |
| Ovarian tumors | |
| Granulosa cell | Highly proliferative |

*Intra-Cranial Lesions:* Patients who have galactorrhea associated with amenorrhea are always suspect of an organic lesion in the region of the pituitary or hypothalamus. This may occur in some patients with a chromophobe adenoma of the pituitary, although other intracranial lesions in this area may also cause a similar picture, as may also certain functional disturbances of the hypothalamus and pituitary. The vaginal smears are generally of the atrophic type or show intermedial proliferation, but the uterus is not generally hyperinvoluted and the degree of vaginal atrophy not so marked as in the Chiari-Frommel syndrome.

**Feminizing Precocious Puberty:** The vaginal cytology smear in patients with various syndromes causing feminizing precocious puberty have been of particular interest to us. Stigmata of precocious puberty, evidenced by breast development, growth of pubic hair, rounding of the body, and uterine bleeding, may occur even in early infancy or early childhood. Indeed, the newborn babe for a few days following delivery may exhibit symptoms of excessive estrogen stimulation, as evidenced by breast swelling, witch's milk and hyperhormonal leukorrhea, with a presence of a highly proliferative type of vaginal smear, which gradually fades to an atrophic smear during the course of the next week or so.

*True Precocious Puberty:* The pathologic conditions which may produce evidence of precocious ovarian function in

children may be classified under the two main headings of true precocious puberty and pseudoprecocious puberty (Table 5). The former refer to those instances in which the ovary is actually stimulated by gonadotrophic hormones, and they therefore go on to ovulation and corpus luteum function, so that in rare instances pregnancy itself has occurred in children as young as five and one-half years of age. These instances of true precocious puberty are generally constitutional in origin; that is, for no known reason the hypothalamic-pituitary mechanism begins to function at an unusually early age, with subsequent response by the ovary. The vaginal smear in such children varies, depending upon the completeness of the syndrome. There may be complete cyclic variations in the vaginal smear, such as is seen in the adult during a normal cycle, or varying degrees of estrogen stimulation in those youngsters who do not go on to ovulate. Indeed, in some instances in which only the breast tissue has been stimulated (telarche) the vaginal smear may be of the atrophic type, indicating that the breast tissue in these children happens to be a more sensitive target organ than the vagina. In such instances the syndrome frequently progresses no further and may even recede. Another less common cause for true precocious puberty is an organic intracranial lesion, such as a tumor in the region of the third ventricle, or encephalitis, which may cause premature stimulation of the hypothalamus. Here the vaginal smear is generally of the proliferative type and ovulation rarely occurs.

*Functioning Tumors:* An interesting chapter in endocrinology is composed of the less common but intriguing cases resulting from functioning tumors of the ovary. The granulosa-cell tumor is the usual one seen in such instances and here the clinical picture is one of progressive somatic and sexual maturation of the isosexual type associated with a growing ovarian mass. The vaginal smear is generally of the highly proliferative type. Although hormone assays also may show increased levels of estrogen in the urine in these cases, the vaginal smear is generally a more sensitive indicator of a relative increased estrogen secretion, particularly in young children. The comas are but very rarely found in children as a cause of this syndrome. Choriocarcinoma, also a very rare lesion, may also cause feminizing precocious puberty, since this tumor secretes large amounts of chorionic gonadotrophin which stimulates the opposite ovary to secrete estrogen and progesterone, thus resulting in a proliferative type of vaginal epithelium. Adrenocortical tumors in females are generally virilizing, thus causing a masculine type of precocious pseudopuberty, but in very rare instances feminization has been noted with this lesion.

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# Sputum Cell Concentration by Membrane Filtration for Cancer Diagnosis:

## A Preliminary Report

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TRYPSIN was used by Haynes[5] and by Farber[3] for liquefaction of sputum. The membrane filtration technic has been employed for concentration of malignant cells from body fluids[1, 2, 6, 11-13] and from blood;[1, 7, 10] it has also been used to concentrate bacteria.[4, 5, 8] The authors have combined the principles of trypsin digestion and membrane filtration in the development of a technic for concentration of exfoliated cells in sputum for cancer diagnosis. By the use of this technic, a higher cell population from a relatively large volume of sputum is available for screening; thus, the accuracy of cancer diagnosis should be increased.

### Procedure

1. Sputum is collected and fixed for four to six hours in isotonic neutral phosphate buffer. The solution is made by dissolving 8.6 grams of Difco Hemagglutination Buffer in one liter of 10 per cent formalin.

2. The fixed specimen is washed twice in isotonic saline solution, and left in the second solution overnight at 4 C.

3. From 2-5 cc. of a representative portion of the specimen is transferred to a 150 cc. polyethylene container. Any dense lumps of sputum are fragmented with scissors.

4. The specimen is digested with 10 cc. of trypsin solution. The latter is made by grinding 10 grams of crude trypsin (4X U. S. P. Pancreatin),* a small amount at a time, with cold 0.1 M Sorensen's buffer at *p*H 7.7. One liter of the mixture is made with added Sorensen's buffer, and centrifuged at 2,000 rpm. for 15 minutes. The supernatant is pipetted in 10 cc. aliquots into test tubes and stored in a deep freeze.

5. A magnetic stirring bar is added to the polyethylene container, and the latter is capped. The mixture is set on a magnetic stirrer which has been placed in an oven at 37 C., and is digested for 30 minutes with moderate agitation. (If lumps of undigested material remain, the digest is centrifuged, the supernatant is discarded, and an aliquot of fresh trypsin is added to the sediment for an additional 15 minutes of digestion. This should practically digest all the lumps; if undigested lumps still remain, they should be separated from the digest and discarded.)

6. The mixture is centrifuged at 700 rpm. for five minutes, the supernatant is discarded, and the sediment is resuspended with vigorous agitation in isotonic saline solution. About 4 cc. of saline per 0.1 cc. of sediment is added. The suspension is now ready for filtration.

7. A piece of Whatman No. 1 filter paper (5.5 cm. in diameter) is placed on a size 6 Seitz filter base, which is fitted to a one liter filtering flask. A membrane filter (4.7 cm. diameter, white, plain, 5 $\mu$ porosity),† a silicone rubber gasket (5.5 cm. outer diameter; 3.7 cm. inner diameter), and a brass funnel having a capacity of 25 cc. and specially made to fit over the Seitz filter

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* Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

† Millipore Filter Corporation, Bedford, Mass.

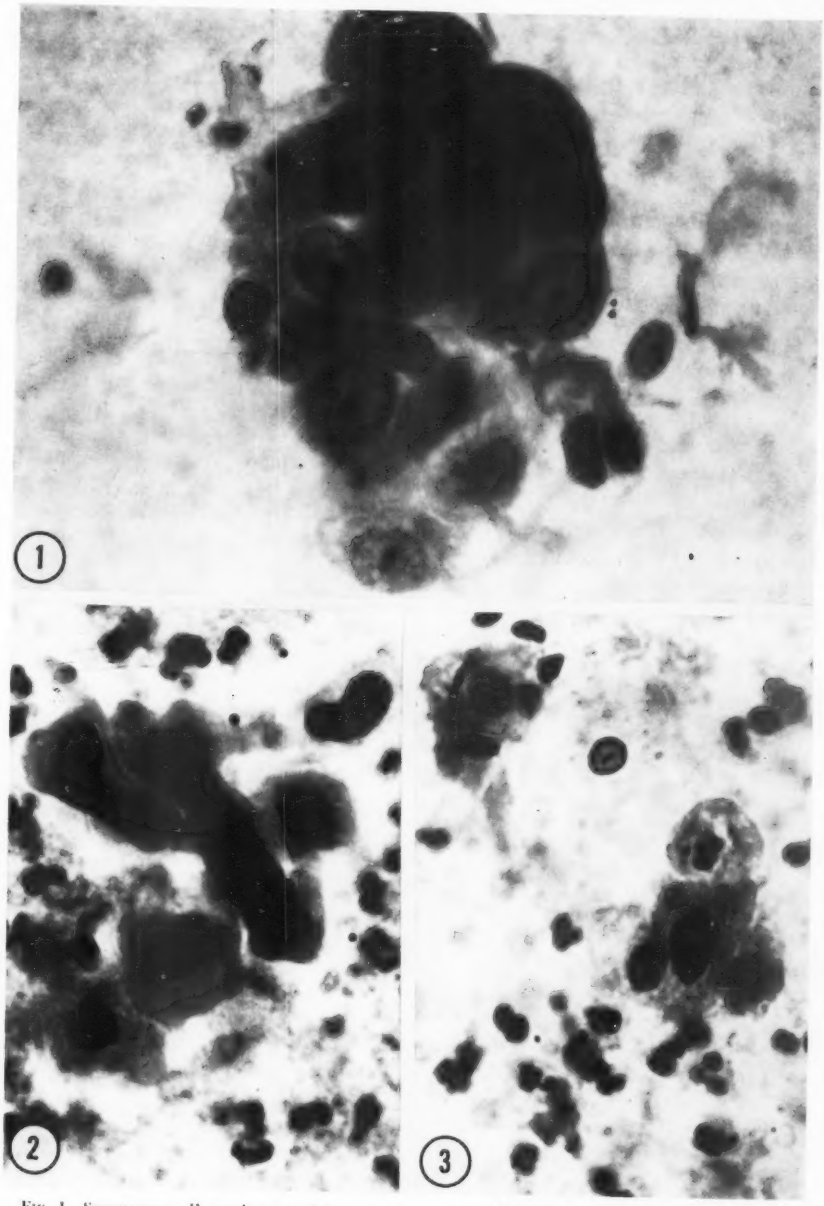



FIG. 1. Squamous cell carcinoma of lung; a group of undifferentiated cells. Membrane filtration technic (x985). FIG. 2, 3. Squamous cell carcinoma of lung; differentiated cells. Membrane filtration technic (x985).

base, are placed over the filter paper in the order mentioned.

8. The membrane filter is wet with saline solution and about 4 cc. of the cell suspension is pipetted onto the filter. With a vacuum pump, slight suction pressure is applied to the filter for a few seconds. If the membrane filter tends to clog, either the cell suspension is diluted or a smaller volume is used; the correct dilution must be determined by trial and error. Usually four membrane filters are used per specimen.

9. While still attached to its filter paper backing, the membrane filter is placed in modified Carnoy's solution (70 parts 95 per cent ethyl alcohol, 25 parts chloroform and five parts glacial acetic acid), suggested by DeWitt, *et al.*,[2] for 30 minutes..

10. Membrane filters are individually mounted in special polyethylene holders†† and hydrated to water.

11. The filters are washed for 20 minutes in running water and then stained by the Papanicolaou method.[9]

12. The filters are dehydrated in alcohol, cleared in xylene, mounted in Permount on 3 × 2 inch slides, and covered with 4.5 × 6.8 cm. No. 1 coverglasses.

### Results and Comments

On the finished slides (Fig. 1-6) the cells are abundant and evenly distributed, without objectionable overlapping and clumping. Fibrinous strands, foreign matter and debris appear almost non-existent. The staining quality of the cells is comparable to that of cells smeared and stained by the conventional method.

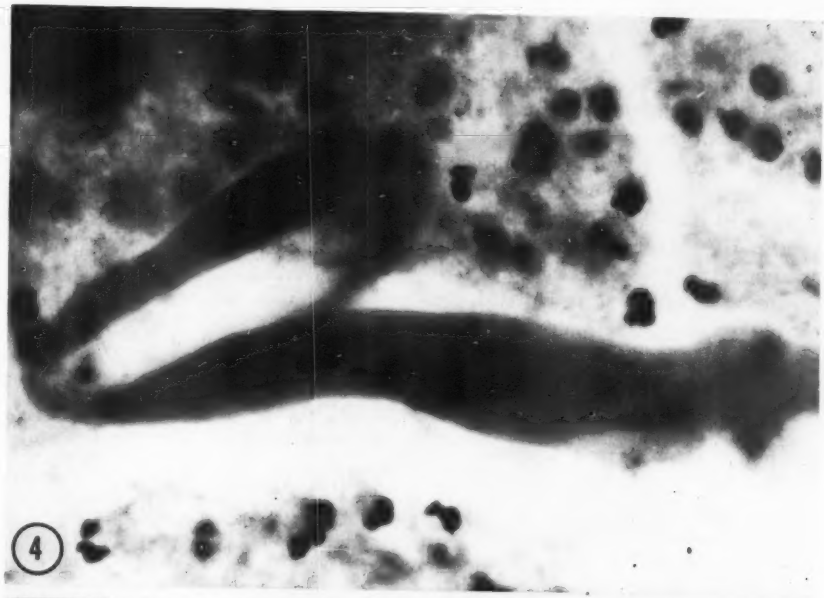
Enzymatic digestion by trypsin, when properly processed, does not alter the morphological details of the exfoliated cells (Fig. 1-6); this is in agreement with the observation of Farber, *et al.*[3] Even the characteristic grouping of some cancer cells is not disturbed by the digestion (Fig. 1). Trypsin, however, does not easily penetrate tenacious clumps of thick sputum. These undigested clumps may contain abundant cells and, if discarded before membrane filtration, cellular material of diagnostic value might be lost. These clumps, however, would clog the membrane filter if allowed to remain in the cell suspension during filtration. For this technical reason, the clumps must be excluded before filtration. Continued effort is being made to solve this problem.

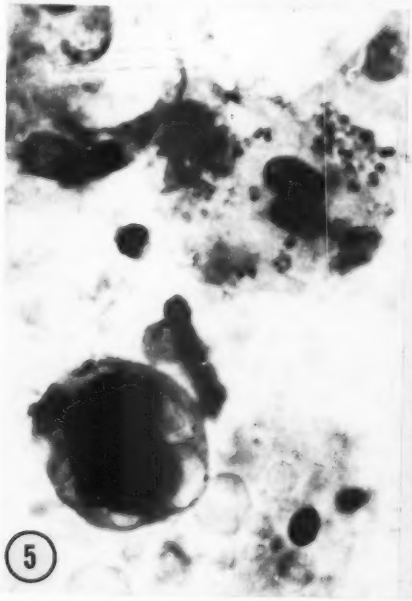
The use of the Seitz filter for membrane filtration, instead of filtering apparatus originally furnished by the Millipore Filter Corporation for this purpose, has the advantage of more rapid and easier operation. The size of the Seitz filter is ideal; it provides more usable area of the membrane filter. Since its metal construction is rigid, the possibility of breakage is lessened.

A two-step fixation method is necessary in this technic. The specimen is first fixed in 10 per cent buffered formalin solution to preserve the cells during collection, then the filtered cells are fixed again in a modified Carnoy's solution to facilitate staining. Without formalin fixation, the cells in sputum would undergo autolysis and lose their cytological integrity, and without Carnoy's fixation the cells would not stain readily. Also, sputum fixed in agents other than formalin resist trypsin digestion. Formalin-fixed specimens are readily mailable in accordance with U. S. postal regulations, this is an advantage in large scale screening of out-patients.

Usually 2-5 cc. of sputum, depending on its viscosity, yields 0.5 cc. of sediment following digestion. This sediment can be collected onto four membrane filters. Cells from a relatively large volume of sputum can be concentrated on a few membrane filters. If 2 cc. of fresh sputum is smeared onto slides by the conventional pull-apart technic, more than 100 slides would be

†† Duke Bontz Company, 8409 2nd Ave., Silver Spring, Md.



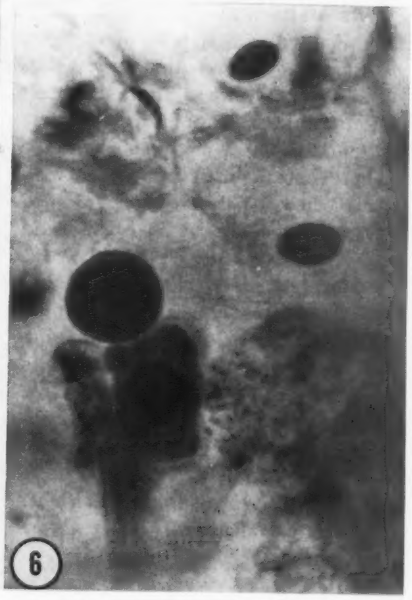




FIG. 4. Squamous cell carcinoma of lung; fiber cells, tadpole type. Membrane filtration technic (x985). FIG. 5. Squamous cell carcinoma of lung; third type of differentiated cell. Membrane filtration technic (x985). FIG. 6. Squamous cell carcinoma of lung; differentiated cells. Membrane filtration technic (x985).

required. Further, the screening area of a single membrane filter is 17 per cent less than that of a regular 3 × 2 inch slide. These facts indicate that the membrane filtration technic is decidedly more efficient than the conventional smear procedure.

It should be noted that this technic is still in the early stages of development. Based upon its theoretical background and our preliminary observations, continued efforts to perfect the procedure are merited. If it proves as successful as anticipated, the technic of trypsin digestion and membrane filtration could be suitable for use in large scale lung cancer screening programs.

## Summary

A method of using trypsin digestion and membrane filtration for the concentration of malignant cells in sputum to increase the accuracy of cancer diagnosis is reported.

Sputum is collected in 10 per cent formalin made up in isotonic neutral phosphate buffer. The fixed specimen is washed and then digested with trypsin. The digest is centrifuged and the sediment is resuspended in normal saline solution. The cell suspension is filtered through a membrane filter mounted upon a modified Sietz type filter. Thereafter, the membrane filter, with the cells attached, is post-fixed in a modified Carnoy's solution, washed in water, and stained by the Papanicolaou method.

On the finished slides cells are abundant and evenly distributed, without objectionable overlapping and clumping. The staining quality of the cells is comparable to that of cells smeared and stained by the conventional technic.

This method is relatively simple and can be performed routinely by laboratory technicians. It should provide much higher accuracy in cancer diagnosis than the conventional smear technic. If our present critical clinical evaluation proves as successful as anticipated, the membrane filtration technic should be suitable for use in large scale lung cancer screening programs.

**Acknowledgments**

The authors wish to express their sincere gratitude for the cooperation and suggestions offered by Drs. H. W. Neidhardt and C. F. Mountain. The technical assistance of Miss Marie Meisenger and Mrs. Janice Bynes is greatly appreciated.

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# A Direct Comparison of Natural and Aerosol Produced Sputum Collected from 776 Asymptomatic Men*

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THE COLLECTION of sputum for cytologic examination has become an important tool for diagnosing malignant tumors of the lung. A common method of sputum collection is asking the patient to expectorate into a jar containing 70 per cent alcohol. This mode of collection results in obtaining adequate sputum; *i.e.*, sputum containing histiocytes or dust cells in approximately 70 per cent of cases. Patients having an acute or chronic cough due to disease of the bronchi or lungs, usually have no difficulty in bringing up material from the bronchi which is adequate for cytologic diagnosis. However, if an improvement is to be made in the resectability and cure rate of lung cancer, the present feeling is that these tumors must be diagnosed in the asymptomatic stage. The following study was undertaken to determine the value of artificial stimulation of the cough reflex in asymptomatic individuals, using a heated hypertonic saline solution, first described by Beckerman, Sproul, and Barach.[1]

In 1958 we collected sputum from 693 asymptomatic males who were undergoing routine physical examinations at a cardiovascular clinic at the Albany Medical College. These men were instructed to inhale a 10 per cent hypertonic saline solution in propylene glycol. Eighty-six per cent of them produced sputum which contained histiocytes. However, we were not able to definitely state whether or not these men would have produced just as adequate a specimen without the artificial stimulation. Therefore, it seemed advisable to collect both naturally produced and artificially produced sputum from the *same* individual and compare the quality of each as regards to method of collection. The experiment was set up in the following manner:

**Sample:** The sample was not selected in a random manner, but consisted of all men attending a cardiovascular health center for a routine physical examination. These men were mostly employees of New York State, with the median age of 48. They were asymptomatic unless suffering from an acute illness which did not prevent their working. Every year these men attend this clinic, where they are given a complete history and physical examination; including PA and lateral chest films, blood counts, cholesterol determinations, and urinalysis. No selection was made as to which of these men were to have sputum collected, but all males going through the clinic were tested.

It was decided to collect the natural specimen first, and then the aerosol, thereby putting an additional burden on the artificial method of sputum collection. Since both specimens were collected on the same day, at close interval, we did not feel that a natural specimen collected after administering the aerosol would be truly representative.

**Apparatus:** 1. A nebulizing machine from The Inhalation Therapy Co. was used. 2.

* Presented at the Eighth Annual Meeting of the Inter-Society Cytology Council, 1960, Chicago, Illinois, U.S.A.

Aided by a grant from the American Cancer Society.

Solution used was 10 per cent saline in 20 per cent propylene glycol.

**Method of Administration:** Each individual was first asked their smoking and coughing habits. A detailed report was taken as to amount and length of smoking habits. The men were then instructed to cough deeply and expectorate into a jar containing 70 per cent ethyl alcohol. Every attempt was made to have them produce a deep cough containing material from the bronchi rather than just saliva. After the natural specimen was collected, they were asked to inhale the nebulized, hypertonic saline solution for at least ten minutes. If coughing was stimulated before this time, they were allowed to expectorate into another jar containing 70 per cent alcohol.

**Slide Preparation:** Two slides were made from each specimen. Each slide was given a random letter; A, B, C, or D. In this way the screener at no time knew which slides contained the natural sputum, or which slides contained the aerosol sputum. The slides were fixed in ether-alcohol and stained by the routine Papanicolaou way.

**Screening:** One Cyto-technician was responsible for screening all of the slides in order to avoid differences in screening technic. The main objective was not only to look for atypical cells or cancer cells, but to attempt an evaluation of the adequacy of the smear. After much deliberation, it was decided to pick out a fairly representative field and to count the number of histiocytes or dust cells noted per high power field. In this way a numerical evaluation of the four slides was made. If no histiocytes, or only a few were found on the entire slide, it was considered inadequate for diagnosis. The slide was considered adequate if it contained more than five histiocytes. Some slides contained as many as forty histiocytes per high power field.

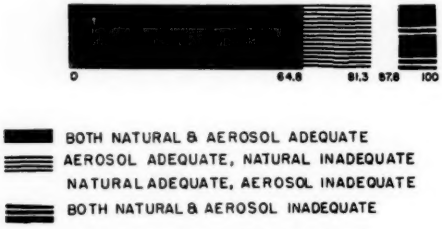


FIG. 1. Percentages of 776 individuals producing sputum specimens.

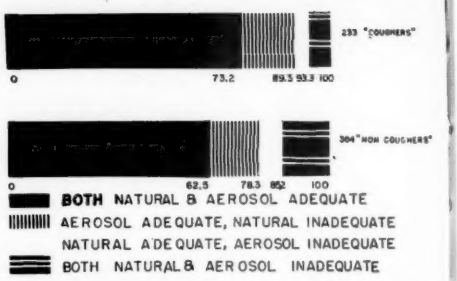


FIG. 2. Adequacy dependent on coughing history.

**Results:** Of 776 individuals studied, 552 (71.1 per cent) produced adequate specimens naturally. Of the 776 individuals, 81.3 per cent produced adequate specimens with aerosol. This gain of 10 per cent, assuming that these were two independent samples, is highly significant. The standard error of such a test would be roughly 1.5 per cent. Of the 776 individuals, 129 (16.6 per cent) produced adequate specimens after aerosol although their naturally produced sputum specimens were inadequate.

There is a highly significant difference between the respective numbers of naturally produced and aerosol aided specimens that were adequate. A Chi-Square test of the difference was made and it was 34. The probability of a value of Chi-Square this large when the null hypothesis is true, is less than .0005.

In Figure 1 you can see that 64.8 per cent of the men produced adequate sputum both naturally and with aerosol as represented by the bar graph. The next group had an aerosol specimen which was adequate, but their natural sputum was

inadequate. This group represents 129, or 16.6 per cent. Six and one-half per cent had inadequate aerosol with natural adequate, and finally 12.2 per cent which were inadequate both ways.

Figure 2 represents the adequacy of the two collection methods on coughing history. The patients admitting to having a cough produced more adequate specimens than those without a cough. But even 85.2 per cent of the noncoughers produced adequate specimens as opposed to 93.3 per cent of the coughers.

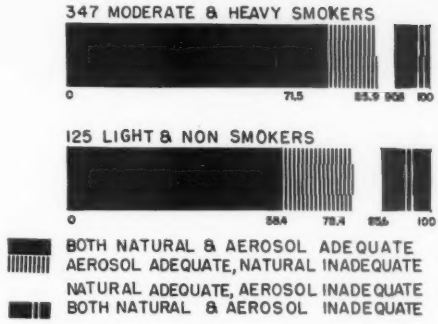


FIG. 3. Adequacy dependent on smoking history.

TABLE 1. *Summary of Measurements for Adequacy of Sputum Specimens (Median No. of Histiocytes Per High Power Field)*

| | No. of individuals | Natural | | Aerosol | |
|---|---|---|---|---|---|
| | | Slide 1 | Slide 2 | Slide 1 | Slide 2 |
| Total | 776 | Few | 4 | 7 | 14 |
| Natural and aerosol adequate | 502 | 9 | 18 | 15 | 21 |
| Aerosol adequate Natural inadequate | 129 | None | None | 9 | 28 |
| Natural adequate Aerosol inadequate | 50 | Rare | 9 | None | None |
| Natural and aerosol inadequate | 95 | None | None | None | None |

TABLE 2. *Chi-Square Tests*

Statistical significance of coughing history
Do you cough now?

| | Observed | | | Expected | | | Chi-square | |
|---|---|---|---|---|---|---|---|---|
| | Yes | No | Total | Yes | No | Total | | |
| Both natural and aerosol adequate | 163 | 190 | 353 | 119 | 204 | 353 | 1.32 | .96 |
| Aerosol adequate, natural inadequate | 36 | 48 | 84 | 36 | 48 | 84 | — | — |
| Natural adequate, acrosol inadequate | 9 | 21 | 30 | 13 | 17 | 30 | 1.23 | .91 |
| Both natural and acrosol inadequate | 15 | 45 | 60 | 25 | 35 | 60 | 4.00 | 2.86 |
| | 223 | 304 | 527 | 223 | 304 | 527 | Chi-square = 11.31 | |
| | | | | | | | $p = < .005$ | |

Statistical significance of sputum production
Have you coughed up any phlegm or sputum?

| | Observed | | | Expected | | | Chi-square | |
|---|---|---|---|---|---|---|---|---|
| | Yes | No | Total | Yes | No | Total | | |
| Both natural and aerosol adequate | 246 | 209 | 455 | 220 | 235 | 455 | 3.07 | 2.88 |
| Aerosol adequate, Natural inadequate | 50 | 61 | 111 | 54 | 57 | 111 | .30 | .28 |
| Natural adequate, acrosol inadequate | 15 | 27 | 42 | 20 | 22 | 42 | 1.25 | 1.14 |
| Both natural and aerosol inadequate | 25 | 63 | 88 | 42 | 46 | 88 | 6.88 | 6.28 |
| | 336 | 360 | 696 | 336 | 360 | 696 | Chi-square = 22.08 | |
| | | | | | | | $p = < .0005$ | |

Figure 3 represents the men who were moderate to heavy smokers; *i.e.*, over one-half pack per day as opposed to the light smokers of less than one-half pack or nonsmokers. As you can see, more adequate specimens were obtained from the heavy smokers than the nonsmokers.

Table 1 shows conclusively that the quality of the sputum collected by the aerosol method is superior to that naturally produced if the number of histiocytes per high power field is the criteria used to determine quality. The aerosol specimens consistently contained more histiocytes per high power field than the naturally produced sputum.

Table 2 shows that more adequate sputum is obtained from patients having a cough or having coughed up phlegm or sputum in the past than could be expected by chance alone.

## Conclusions

The following conclusions were reached by a statistical evaluation of the results.

1. A highly significant increase in adequate specimens were obtained from the same individuals using artificial stimulation of the cough reflex by a heated 10 per cent saline solution.

2. A significant increase was also obtained from patients admitting a cough or having previously coughed up sputum.

3. Men who were still smoking also had a significant increase, but there was no statistically significant increase in aerosol sputum as opposed to naturally produced sputum in heavy or light smokers.

A percentage comparison of atypia was made. Eighty-six per cent of the men admitted to using tobacco, and 30.1 per cent of the subjects were heavy smokers (over one package per day), and 100 per cent of the atypical smears occurred in the heavy smoker and moderate smoker group. None of the light or nonsmokers had atypical cells.

There was only one case of proven lung cancer in this group, which was detected by chest x-ray. The smears were negative. On section of the lung, it was found to be a peripheral tumor with no bronchial connection.

No false positives were given. Several men had markedly atypical cells and negative x-rays. They are being closely followed by repeated sputum examinations.

We have now proven to our satisfaction that aerosol stimulation is more likely to produce an adequate specimen containing more histiocytes per high power field than a naturally produced sputum, and we will continue collecting only aerosol specimens for the next three years in these same individuals. Our hope is to detect the early asymptomatic lung cancers and to evaluate the atypical cells found in heavy smokers.

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# Exfoliated Cancer Cells in the Bronchi of Lungs Resected for Bronchogenic Carcinoma*

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## Introduction

The purpose of this investigation was to determine the extent of dissemination of exfoliated malignant cells and their anatomical distribution within the bronchial tree of patients with bronchogenic carcinoma.

Data of this nature could yield information relating to basic physiology of the respiratory tract, such as variations in the routes of propulsion of secretions, and exchanges of secretions between various subdivisions of the respiratory tract at different levels in the bronchial tree. It was also hoped that this information might help explain why cytologic studies of sputum and bronchial secretions frequently are not uniformly positive in patients who have pulmonary neoplasms. Another consideration was that data of this type might be of help in evaluating the hypothesis that free-floating neoplastic cells within the bronchial tree could initiate secondary growths in other areas of the respiratory tract. This hypothesis has been debated since it was suggested by Moxon in 1869. Although it does not have widespread acceptance, Furth[1] was able to initiate growth of a variety of tumors in the lungs of mice by intranasal instillation of neoplastic cells; and Willis,[2] in reviewing a number of cases of human pulmonary neoplasms attributed to aerial metastasis, concluded that it is possible that some dissemination of tumors within the lungs may occur by implantation of neoplastic cells on apparently intact epithelial surfaces.

## Material and Methods

The subjects in this investigation were 34 patients at M. D. Anderson Hospital who underwent pneumonectomy for bronchogenic carcinoma. Thirty of the patients were male, and four female. Their ages ranged from 43 to 78, with a median age of 60. Right pneumonectomy was performed on 15 of the patients and left pneumonectomy on 19.

At the time of pneumonectomy, immediately on removal of the surgical specimen, dissection was carried out to the level of

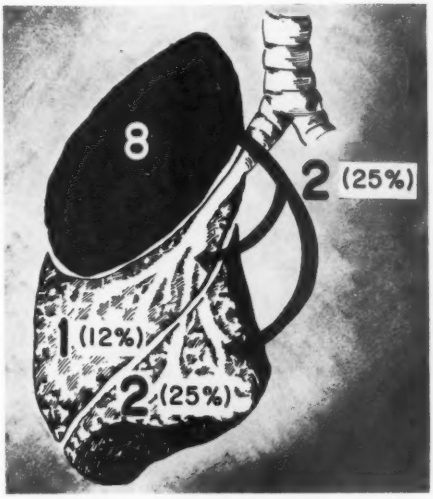


Figure 1.

* Presented at the Eighth Annual Meeting of the Inter-Society Cytology Council, 1960, Chicago, Illinois, U.S.A.

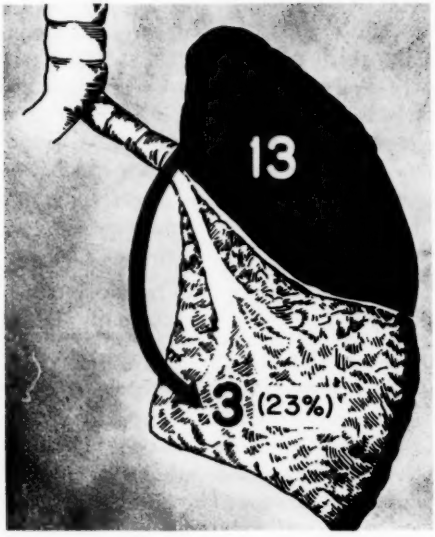


FIGURE 2.

the segmental bronchi. The bronchi were opened and secretions were taken from the bronchial mucosa. The smears were stained by the Papanicolaou method, and screened for malignant cells.

In addition to the data obtained from the direct smears from resected lungs, preoperative sputum cytology reports were available on 29 of the 34 patients, and follow up information was obtained relating to survival and recurrences.

## Results

The intrapulmonary sites of the primary tumors and the intraluminal distribution of exfoliated malignant cells in the uninvolved lobes are illustrated in Figures 1 through 6.

Eight of the patients had the primary tumor located in the right upper lobe. Two of the eight, or 25 per cent, had exfoliated malignant cells present in other lobes of the right lung. One of these two patients had positive smears in the bronchi of both the middle lobe and the lower lobe, while the other had positive smears in the lower lobe only.

In 13 patients the primary tumor was located in the left upper lobe. Three of these, or 23 per cent, had positive smears in the lower lobe.

None of the 34 patients in this group had primary tumors in the middle lobe. The primary tumor was located in the right lower lobe in four patients. Three of these, or 75 per cent, had positive smears in the uninvolved lobes. Two of the three had malignant cells in both the upper lobe and the middle lobe, while one had malignant cells in the upper lobe only. Four of the primary tumors were located in the left lower lobe. Two of these, or 50 per cent, had positive smears in the left upper lobe.

Three patients had the primary tumor located in the right mainstem bronchus. Two of these, or 67 per cent, had positive smears in one or more of the lobes of the right lung. One of the two demonstrated

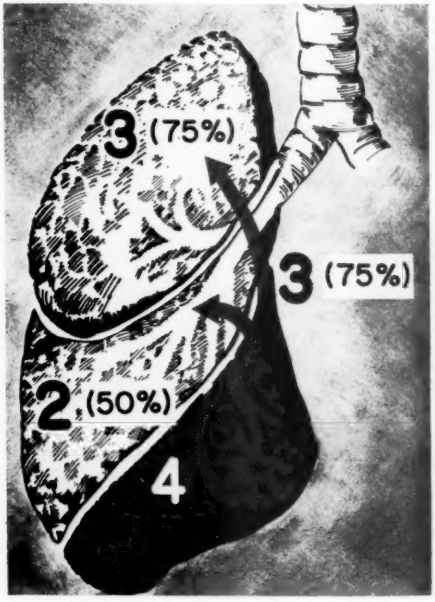


FIGURE 3.

malignant cells in all three lobes, while the other had malignant cells in the upper and middle lobes only.

In the remaining two patients, the primary tumor was located in the left mainstem bronchus. One of these, or 50 per cent, had spread of the malignant cells, involving both lobes of the left lung.

Analysis of the distribution of exfoliated malignant cells by the individual bronchopulmonary segments revealed no consistent pattern of distribution with one exception: in each of the seven cases where there was spread of exfoliated malignant cells to a lower lobe from a primary located elsewhere, malignant cells were always found in the superior segment, even when the smears from the other segments of the lower lobe were negative.

Table 1 illustrates the distribution of primary tumor sites in relation to the dissemination of malignant cells in the uninvolved lobes. Since the distributions were very similar in the left and right lungs, the data for the two sides are combined in the tables.

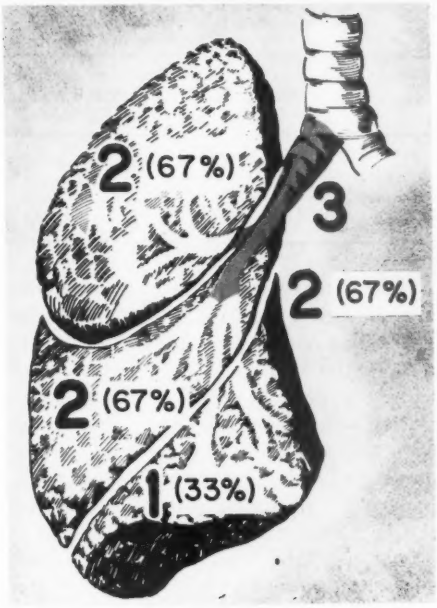


FIGURE 5.

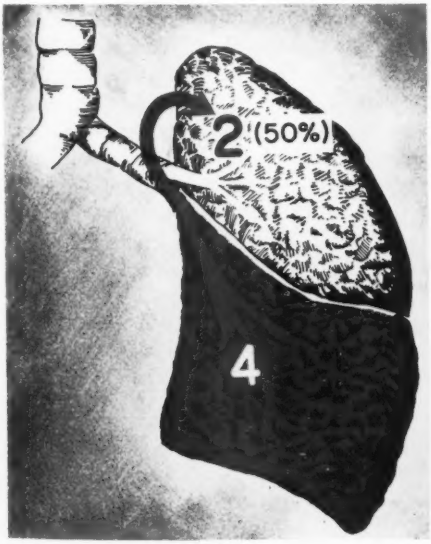


FIGURE 4.

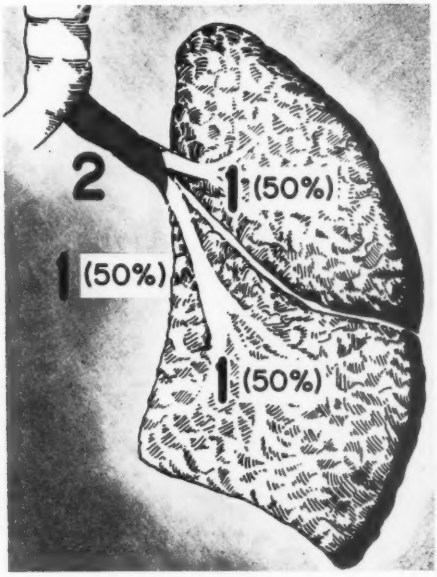


FIGURE 6.

TABLE 1. *Distribution of Primary Tumor Sites in Relation to Dissemination of Malignant Cells*

| Location of primary tumor (number) | | Positive smears in other lobes Number-% | |
|---|---|---|---|
| Upper lobes | 21 | 5 | 24 |
| Lower lobes | 8 | 5 | 62 |
| Mainstem bronchi | 5 | 3 | 60 |
| All | 34 | 13 | 38 |

Twenty-four per cent of the patients who had the primary tumor in an upper lobe had dissemination of exfoliated malignant cells to the bronchi of uninvolved lobes, while ,62 per cent of the lower lobe primaries, and 60 per cent of the mainstem bronchus primaries had dissemination to uninvolved lobes. A comparison of the upper lobe primaries with all other primaries yields a t-ratio of 2.32, which is significant at the 5 per cent level of confidence. Thus, the upper lobe primaries less frequently show dissemination of malignant cells to uninvolved lobes than do all other primaries. Of the total group of 34 patients, 38 per cent had dissemination of malignant cells to uninvolved lobes.

Table 2 compares the various primary tumor sites in terms of the findings on preoperative sputum cytology. Forty-one per cent of the patients who had upper lobe primaries had sputum specimens reported as positive prior to surgery, as compared with 57 per cent of the patients who had lower lobe primaries and 100 per cent of the patients who had mainstem bronchus primaries. A comparison of upper lobe primaries with mainstem bronchus primaries yields a t-ratio of 4.96, which is significant at the 1 per cent level of confidence. The lower lobe primaries did not differ significantly from the other two groups of primaries. Of the total group of 29 patients who had preoperative sputum cytology reports, 55 per cent were reported positive, 10 per cent suspicious, and 35 per cent negative.

In Table 3, the various histologic types of tumor are compared in terms of extent of intrabronchial dissemination of malignant cells. Forty-two per cent of the squamous cell carcinomas, and 40 per cent of the adenocarcinomas, had positive smears in uninvolved lobes of the lung, while none of the undifferentiated carcinomas had positive smears in uninvolved lobes. Although there were only three undifferentiated carcinomas in the group studied, they differ significantly from the others in the degree of dissemination of malignant cells, as reflected in a t-ratio of 4.72, significant at the 1 per cent level of confidence.

In Table 4 the tumor sizes are compared in patients who had dissemination of malignant cells to uninvolved lobes and patients who did not have dissemination. The median diameter was 3.5 cm. in the patients who did have dissemination, while the patients who did not have dissemination had a median tumor diameter of 6.0 cm. The difference between the two groups is not statistically significant, as reflected in a Kruskal-Wallis H value of 3.73.

TABLE 2. *A Comparison of Primary Tumor Sites and Preoperative Sputum Cytology*

| Location of Primary tumor | Preoperative sputum cytology Positive | Suspicious | Negative | Total |
|---|---|---|---|---|
| Upper lobes | 7 (41%) | 2 (12%) | 8 (47%) | 17 (100%) |
| Lower lobes | 4 (57%) | 1 (14%) | 2 (29%) | 7 (100%) |
| Mainstem bronchi | 5 (100%) | 0 (0%) | 0 (0%) | 5 (100%) |
| All | 16 (55%) | 3 (10%) | 10 (35%) | 29 (100%) |

TABLE 3. *Distribution of Cell Types in Relation to the Dissemination of Malignant Cells*

| Cell type (number) | | Positive smears in other lobes Number-% | |
|---|---|---|---|
| Squamous | 26 | 11 | 42 |
| Adenocarcinoma | 5 | 2 | 40 |
| Undifferentiated | 3 | 0 | 0 |
| All | 34 | 13 | 38 |

TABLE 4. *Size of Primary Tumor in Relation to the Dissemination of Malignant Cells*

| | Number | Tumor size—cm. Range | Median |
|---|---|---|---|
| Positive smears in other lobes | 13 | 2.5-10.0 | 3.5 |
| No positive smears in other lobes | 21 | 2.4-15.0 | 6.0 |
| All | 34 | 2.4-15.0 | 5.6 |

## Summary and Conclusions

The results of this study indicate that exfoliation of malignant cells from primary pulmonary neoplasms is more common in lesions of the lower lobes and mainstem bronchi than in upper lobe lesions, with respect to preoperative sputa, as well as dissemination within the bronchial tree.

The reason for this difference is not apparent in available data, but it is possible that in coughing, greater propulsive forces in the lower lobes aid in dissemination of malignant cells from the area of the primary lesion, both into sputum and into uninvolved lobes of the lung. Another factor to be considered in the greater spread of exfoliated cells from lower lobe lesions is the fact that the normal upward pathway of secretions past the orifice of the short upper lobe bronchus brings them in close proximity to the segmental bronchi of the upper lobe.

Whatever the mechanism, it does appear that there can be some exchange of secretions between different subdivisions of the lung, and that digression of the secretions from their normal pathway at the level of the secondary bronchi may extend at least as far as the tertiary bronchi.

At the present time the follow ups on these patients are not of sufficient duration, and data from related studies are not available, to permit inferences relating these results to the occurrence of secondary growths by implantation of exfoliated malignant cells.

The trends demonstrated in the data suggest that more detailed studies of intrabronchial distribution of exfoliated malignant cells might contribute information on the problem of the occurrence of some secondary intrathoracic growths in bronchogenic carcinoma, and at the same time contribute to the understanding of some aspects of basic pulmonary physiology and sputum cytology.

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# Cytologic Diagnosis of Cytomegalic Inclusion Disease*

## A Case Report

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### Introduction

CYTOMEGALIC Inclusion Disease is the result of infection by the salivary gland virus which produces a characteristic cytologic picture consisting of cellular enlargement and both nuclear and cytoplasmic inclusions. It is primarily a disease of infants, frequently fatal. Diagnosis of this condition can be made by the examination of cytologic preparations of several types of specimens, especially urinary sediment, for the alterations of the cells infected by this virus are accepted as being pathognomonic.

Until 1952, cytomegalic inclusion disease was a condition of interest mainly to the pathologist, but with improved diagnostic methods it has become apparent that, although a generalized infection before or shortly after birth is usually fatal, it does not always result in death. We found reports of seven infants in whom the diagnosis was made during lifetime and who survived.[2, 8, 9, 13, 14, 21] Typical inclusion cells were found during the acute stage in the urine of all but one of these cases, and in one of them the abnormal cells were also demonstrated in a gastric cell block. In most of those who survive there are some sequelae, frequently associated with the central nervous system. Five of the seven patients mentioned had varying degrees of residual neurological disorders. Reports of only two cases of infants with generalized cytomegalic inclusion disease have been found in which there were no symptoms after the age of about one year.[2, 21] Our patient, in whom the diagnosis was made on the third day of life and who is now six months old, also shows no sequelae other than hepatomegaly and is doing well.

### Historical Data

Jesionek and Kiolemenoglou,[10] in 1904, first reported finding cytomegalic inclusion cells in the kidneys, lungs, and liver of an eight month fetus. They thought that the cellular alterations might be attributed to protozoa. Prompted by that report, Ribbert described similar findings which he had noted first in 1881 in the kidneys of a stillborn infant. In recent years, there has been a rapidly increasing number of reports of the typical inclusion-bearing cells in various organs of fetuses, infants, young children and a few adults.[4, 5, 7, 20, 22, 23, 27] In 1950, Wyatt, *et al.*,[26] suggested that, since the inclusions were so often found in the renal tubules of infants, study of the urinary sediments might reveal the infected cells before the infant's death. This was verified in 1952 by Fetterman[6] who succeeded in finding the characteristic cells in a urinary specimen of an infant who lived for only four days. Other cases have since been diagnosed prior to the patient's death by recognition of the infected cells not only

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in urinary sediments but also in gastric washings,[3, 9, 18] lung tissue,[8] liver biopsy,[24] and "subdural tap."[14]

Using tissue culture technics, Margaret Smith[19] in 1956 isolated the human salivary gland virus from the submaxillary gland of an infant who died of adrenal carcinoma and also recovered it from the kidney of another infant dying of generalized cytomegalic inclusion disease. Isolation of the virus from the urine of patients has been shown to be possible whether inclusion-bearing cells are found in the sediment or not.[17] Complement-fixing antibodies against the virus have also been demonstrated.[17, 24]

### Incidence and Clinical Manifestations

Reports based on routine autopsies have indicated an incidence of the infected cells in the salivary glands of 10-32 per cent of infants and young children.[20] Because only a small percentage of these cases have shown similar changes in the viscera, it would seem that the virus frequently occurs in the salivary glands in a latent stage and only rarely spreads from that site. Dissemination leads to the generalized form of the disease which is dangerous particularly to the newborn. In these patients, the more common clinical manifestations of generalized cytomegalic disease include below-average birth weight, hepatosplenomegaly, hemolytic anemia, thrombocytopenia, hemorrhagic phenomena, jaundice, and cerebral calcification. Chorioretinitis is sometimes present. This and cerebral calcification may lead to difficulty in the differential diagnosis between cytomegalic inclusion disease and congenital toxoplasmosis. Infection of the salivary glands of older children and adults as a rule is of little consequence although dissemination, leading to the generalized form of the disease, may occur in patients who are debilitated by other diseases.[7, 15, 16, 23]

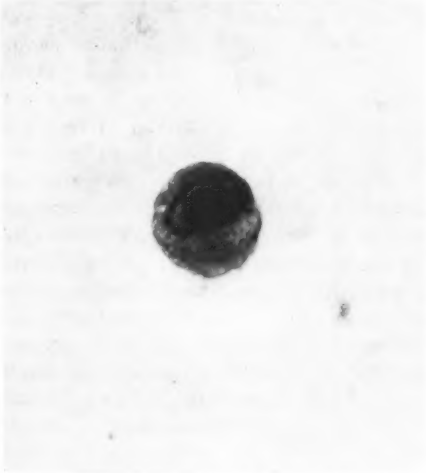

FIG. 1. Typical inclusion-bearing cell found in the urine of the patient (×800).

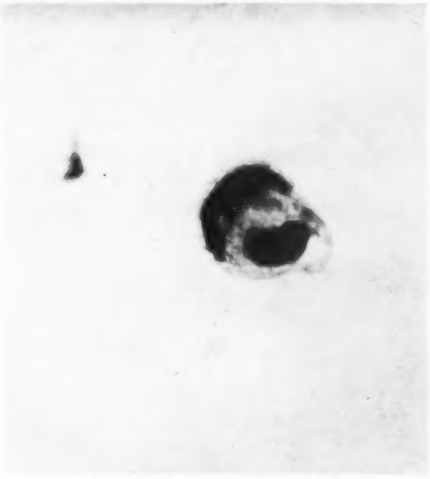

FIG. 2. Cell showing cytoplasmic inclusions (×800).

### Case Report

Our case is that of a Negro male infant weighing 3,440 grams, born on March 22, 1960 after 38 weeks of gestation terminated by normal labor and spontaneous delivery. The mother is a 42-year-old gravida 2, para 2, whose pregnancy was complicated only by first trimester bleeding and hydramnios. The sibling of our patient, a seven-year-old normal girl, had "measles" two weeks before this child was

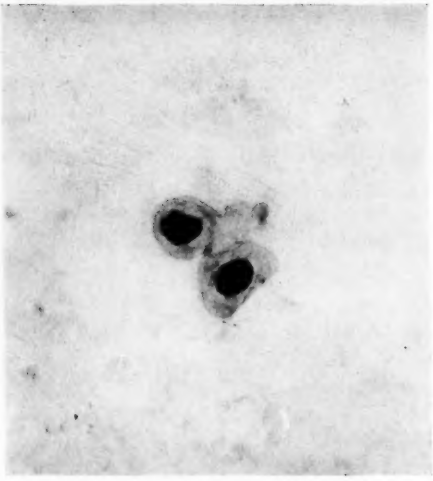

FIG. 3. Normal transitional cells for comparison with Figures 1 and 2 (×800).

born and the mother simultaneously had a bad "chest cold," but this was subsiding at the time of delivery. The mother's blood type was O, Rh positive, with a negative serological test for syphilis. At birth, the infant's cry was good and respirations were established immediately. It was in no distress and the only positive physical findings were a generalized petechial and purpuric rash and enlargement of both liver and spleen, the liver being palpable 3-4 cm. and the spleen 3 cm. below the costal margin. There was no jaundice. The infant's blood type was O, Rh positive, and the Mazzini test was negative. During the first two weeks of life, the hemoglobin ranged from 14.3 to 15.9 Gm., the erythrocyte count was between 4.76 and 5.18 million, and there were two nucleated red blood cells per 100 white blood cells. The WBC varied from 11,200 to 19,750 with an essentially normal differential count. Initially, the platelets were counted at 30,000 to 50,000 per cubic millimeter, but rose to 280,000 in the third week. A maternal blood smear revealed adequate platelets.

The child's total bilirubin, at the age of two weeks, was 1.5 mg. per cent, of which 0.8 was direct and 0.7 was indirect. Skull x-rays were normal, showing no evidence of intracranial calcification. The first urinary specimen interpreted as being positive for cytomegalic inclusion cells was obtained at the age of three days and similar cells were demonstrated in subsequent specimens on several occasions. The infant, which was in no distress at any time, improved steadily during its stay in the hospital. On the second day of life, the petechial eruptions had markedly diminished, and they disappeared completely at the age of about one week.

The spleen decreased markedly in size and the infant gained weight steadily. Except for a single dose of gamma globulin at the age of one week, no therapy was given. Discharged from the hospital at three weeks of age, he continued to do well at home. He was seen in the Pediatric Follow Up Clinic at monthly intervals, and development during this time appeared quite normal. He was alert and active. Jaundice has never been evident and the liver is now 3.0 cm. below the costal margin. The spleen is no longer palpable. Examination of the fundi showed no sign of chorioretinitis. Cytological examination of the child's urine at the age of 12 and 22 weeks failed to show any cytomegalic cells, and samples of the mother's and sibling's urine and the mother's saliva were also negative. A viral culture from the patient's urine was positive, producing the characteristic inclusions in fibroblasts, and complement-fixing antibodies against the salivary gland virus were demonstrated in the patient's serum.

## Cytology

The characteristic cell, which is the hallmark of cytomegalic inclusion disease and which is found in the tissues as well as in exfoliated material, is a large cell measuring between 20 and 35 $\mu\mu$ in diameter. The nucleus is also large, measuring from 10 to 15 $\mu\mu$ across, and contains a dense homogeneous inclusion which may occupy the major portion of the nucleus. The inclusion is usually oval and most often is basophilic but it may be eosinophilic. Its border is quite smooth. The visible portion of the nucleus is largely vesicular or slightly granular and appears as a halo surrounding the inclusion. The nuclear membrane is frequently distinct, staining darkly. The nuclear chromatin tends to occur in clumps closely applied to the nuclear membrane. In general appearance, the cell has been described as being similar to an "owl's eye." In some of these cells, but not in all, cytoplasmic inclusions are also found. These are multiple and much smaller than the intranuclear inclusions, ranging in size from what is just visible up to 3-4 $\mu\mu$ across. They have been observed only in cells which contain the intranuclear inclusions, are basophilic, and tend to gather in a crescentic arrangement peripherally in the cytoplasm. Electron microscopic studies

have indicated particles thought to be virus in both nuclei and cytoplasm.[12]

## Pathology

At autopsy, the pathognomonic feature is the presence of the distinctly enlarged cells containing intranuclear and cytoplasmic inclusions. In addition to the salivary gland, the organs most frequently affected are the kidney, lung, pancreas, gastro-intestinal tract, and brain, although many other organs may also show the cellular characteristics of this viral infection. In the kidney, which is the organ of most interest from the standpoint of exfoliative cytology, the inclusion-bearing cells are found chiefly in the convoluted tubules, and more rarely in the loops of Henle and the collecting tubules. They have also been described in the glomeruli. The affected tubules are dilated and the large cytomegalic cells stand out very prominently. It is interesting that transitions are almost never seen between the cells of the normal tubular epithelium and the cytomegalic cells. Inclusion-bearing cells may fragment or become detached from the basement membrane and be found lying free within a renal tubule. Although infected cells may be found in tissues in the absence of an inflammatory reaction, a focal mononuclear infiltrate in the interstitium of the involved tissue is often described. This is especially true of the brain which may exhibit a necrotizing granulomatous encephalitis with extensive calcification.[11, 25]

## Summary

The patient, whose bout with cytomegalic inclusion disease is herein reported, was a male infant three days old when examination of the urine first disclosed the presence of the characteristic enlarged cells with both intranuclear and intracytoplasmic inclusions. Clinically, he exhibited generalized petechiae and purpura, hepatosplenomegaly, and thrombocytopenia, but there was no jaundice and no intracranial calcification. Without treatment, except for one dose of gamma globulin at the age of one week, he improved steadily and the urine became negative for inclusion-bearing cells before the twelfth week of life. At six months of age, he showed no sequelae other than hepatomegaly and at last report was doing well. The absence of adverse residual effects is somewhat unusual. A brief historical review, a short description of the clinical and pathological aspects of this disease, and illustrations of the cytologic findings are presented.

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# Exfoliative Cytology in Non-Malignant Conditions of the Upper Intestinal Tract*

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## Introduction

THE CLINICAL value of exfoliative cytology in differentiating benign from malignant lesions of the upper gastro-intestinal tract has been well documented during the past decade.[1, 6, 7, 10-12, 17] Pretreatment diagnosis of malignancy of the esophagus and stomach approaches histologic appraisal if the examination is properly performed and the cells expertly interpreted (85-98 per cent correct diagnosis). It is the only method short of histologic examination of establishing a preoperative diagnosis of malignant lymphoma.[13] Exfoliative cytology is even more effective in excluding suspected malignancy of these organs (98-100 per cent correct benign diagnosis).

The diagnostic usefulness of cytology of the duodenum and of the organs it drains is far less. With extraordinary efforts in cell collection and great interpretive skill a correct diagnosis of malignancy is obtained in approximately 50-60 per cent of patients with cancer in this area and this rarely affects the curability of the lesion. Negative results mean nothing.

Cytology is effective in differentiating benign from malignant lesions of the esophagus and stomach whether or not they are diagnosed by other methods such as by x-ray or endoscopy; its role in the diagnosis of metaplasia, atrophy, inflammation, etc. is less specific. The only certain way of making a histologic diagnosis such as gastritis is by examination of tissue.[2, 5, 18] Cellular alterations which have been ascribed to "gastritis"[16] are not specific and do not necessarily reflect this confused histologic entity.

Cellular atypias are encountered in upper gastro-intestinal cytology which suggest certain metabolic and histologic abnormalities; they are not diagnostic in themselves. Graham and Rheault[4] first reported alterations in the squamous epithelium, columnar cells and histiocytes exfoliated from the lung, esophagus, stomach and vagina in untreated pernicious anemia. These changes consisted of enlargement of the nucleus and cytoplasm, multi-nucleation and abnormal distribution of chromatin in the nucleus. They also noted that specific treatment caused the squamous epithelium to revert to normal. Massey and Rubin[8] further extended these observations and were able to demonstrate permanent abnormalities in the gastric columnar cells in all 21 of their patients with treated pernicious anemia studied by gastric cytology. Massey and others[3, 9] have found similar changes associated with simple achlorhydria, gastric ulcer, gastric and esophageal carcinoma, and tropical sprue. Temporary cellular alterations have been noted in the gastric cytology in untreated megaloblastic anemia of pregnancy and folic acid deficiency which are indistinguishable from malignancy.[14] It is inadvisable to make a cytologic diagnosis of malignancy until these specific nutritional deficiencies have been corrected.

The majority of cells exfoliated in treated pernicious anemia are morphologically nor-

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mal. A few large columnar cells are regularly found in all patients with pernicious anemia although more than one examination may be required to recover them.

The typical large "bland" cell[8] has a delicate nuclear membrane which may be folded or creased. The chromatin is distributed in fine aggregates on a relatively empty background. Nucleoli are small or not seen. The nucleus and cytoplasm enlarge proportionately maintaining a normal ratio. The cytoplasm of the large cell varies from a finely granular eosinophilic appearance to a mucus containing goblet cell with a basally located nucleus. Intestinal absorptive cells with brush borders are frequently seen.

Large "active" cells[8] are seen in untreated pernicious anemia and nutritional folic acid deficiency. These cells have clumped, irregularly distributed nuclear chromatin within heavy irregular nuclear membranes suggesting rapid growth. They may closely resemble malignant cells, however a normal nuclear to cytoplasmic ratio is maintained in most of them. In this laboratory, large "active" cells persisting in treated pernicious anemia have been found more consistently in patients who have polyps on gastroscopic examination.[14]

For purposes of discussion, the preceding descriptions have been limited to pernicious anemia. It must be stressed that while the cellular alterations described are characteristic of pernicious anemia, they are not pathognomonic and occur in many other situations.

## Material and Methods

During the past six years, 717 patients have been examined by gastric cytology and 145 by esophageal cytology. The original chymotrypsin lavage technic[15] or a simplified modification[1] has been used to collect the gastric cells. Simple lavage with Ringer's solution has been used to collect esophageal specimens.

There were 135 patients with gastric malignancy and 60 patients with esophageal malignancy. The remaining patients had benign conditions which were proven to be so histologically or by their subsequent clinical course.

Included in the benign lesions were 113 patients with treated or untreated adult pernicious anemia, many of whom had had multiple cytologic examinations. The untreated pernicious anemia patients had mouth, nasal and conjunctival scrapings in addition to esophageal and gastric cytologies. Six patients with pernicious anemia had developed gastric carcinoma.

The cases were reported as benign or malignant. A suspicious or doubtful category was not used. The presence of large squamous cells, histiocytes, columnars, intestinal absorptive cells, goblet cells and inflammatory cells was recorded. The "bland" or "active" nature of the cells was noted.

## Findings

Atypical enlarged squamous cells with abnormal nuclear chromatin distribution and frequent multi-nucleation were observed to be exfoliating from the mouth, pharynx, esophagus and lung in all patients with untreated pernicious anemia, nutritional folic acid deficiency anemia and untreated megaloblastic anemia of pregnancy. They were also seen in two patients with untreated idiopathic sprue, in one patient with a "blind loop" syndrome and in one with multiple small bowel diverticulosis. A group of elderly patients with no detectable nutritional deficiencies had large squamous cells. In those patients with specific nutritional deficiencies, the squamous cells became normal with specific treatment.

Large gastric columnar cells of varying degrees of "activity" were observed in 282 patients in this highly selected series (Table 1). Four of 113 patients with treated pernicious anemia did not have large cells in

one examination but no repeat examination was possible in these. It is of interest that six patients with gastric carcinoma and pernicious anemia had cells ranging from large "bland" columnars through increasing "activity" to frank malignancy. Special efforts to recover large cells in the patients with gastric malignancy were not made. It is probable that more than 30 of these patients would have had large cells if repeat cytologies had been performed to detect them.

The miscellaneous nutritional deficiency group includes patients with untreated idiopathic sprue, multiple small bowel diverticulosis and enteroenteric fistula and hepatic cirrhosis with macrocytic anemia. They exfoliated large cells everywhere.

## Discussion

Large atypical benign cells have been described in a variety of situations. The heterogeneity[1] of the preceding group of patients testifies to the non-diagnostic nature of the macrocyte. The findings of generalized macrocytosis in untreated pernicious anemia and folic acid deficiency is evidence that these deficiencies are general and not limited to the hematopoietic system. With specific therapy, the macrocytosis of all the exfoliating cells except the gastric columnar cells in adult pernicious anemia reverts to normal; this lack of change reflects the permanent gastric mucosal atrophy invariably observed in these patients.

The most constant finding, exclusive of the reversible nutritional deficiencies, associated with the presence of large gastric cells in this entire series has been gastroscopic or biopsy evidence of atrophy and intestinalization. Because of the patchy distribution of gastritis or atrophy it is very difficult to exclude or include this diagnosis short of examining a total gastrectomy specimen exhaustively. "Gastritis" by histologic examination has been a less constant finding when large cells exfoliated. The presence of polymorphonuclear leukocytes, plasma cells, lymphocytes and phagocytosing cells were not reliable in diagnosing histologically proven gastritis. This is not surprising because inflammatory cells in the gastric contents often are nongastric in origin.

TABLE 1. *Patients with Large Benign Cells*

| | |
|---|---|
| Adult Pernicious Anemia | 113 |
| Benign gastric ulcer | 40 |
| Gastric carcinoma without pernicious anemia | 30 |
| Gastric atrophy, achlorhydria | 23 |
| Misc. nutritional deficiencies | 17 |
| Gastritis by biopsy | 16 |
| Gastric polyps | 16 |
| Folic acid deficiency anemia | 11 |
| Distant malignancy | 8 |
| Sub-total gastrectomy | 4 |
| Gastric lymphoma | 3 |
| Gastric eosinophilic granuloma | 1 |

It is the opinion in this laboratory that the large columnar cell usually represents gastric atrophy with or without inflammation. The finding of macrocytosis at all exfoliating surfaces suggests untreated vitamin $B_{12}$ or folic acid deficiency, although the findings are non-specific. The diagnosis of pre-anemic pernicious anemia has been confirmed by appropriate vitamin $B_{12}$ absorption tests in a patient with generalized macrocytosis but no anemia.[14] Generalized macrocytosis is not infrequently seen in the aged; whether this is a change due to senescence or malnutrition is unsettled.

While large cells have been observed frequently in benign gastric ulcer, we do not think a diagnosis of ulcer can be made on this basis. Gastric ulcer patients have all had gastroscopic or histologic evidence of atrophy.

## Summary

1. Large atypical squamous and columnar cells have been observed in the gastric contents of 282 patients with a wide variety of disease.

2. Previous observations that large columnar cells are regularly found in treated

and untreated adult pernicious anemia have been confirmed and extended.

3. It is suggested that the common denominator of the presence of the large columnar cell in the gastric cytology is the presence of gastric atrophy, exclusive of reversible Vitamin $B_{12}$ or folic acid deficiencies.

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# The Detection of Early Cancer of the Mouth by Exfoliative Cytology

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ASYMPTOMATIC abnormalities of the mouth are very common. Usually, their character is obvious, but some of these innocent-appearing lesions represent early cancer. The clinical diagnosis of mouth cancer at this early stage of development is completely unreliable and it is not practical to biopsy every minimal oral abnormality.

The successful application of exfoliative cytology to the detection of uterine cancer suggested that cytology might be equally effective for the detection of early asymptomatic cancer of the mouth. The medical literature contained few references to the cytology of oral mucosa,[1-10] but it was known that scrapings from the surface of clinically recognizable oral cancer would yield aberrant cells suggestive of carcinoma.

The investigation reported here explored two questions. First: How reliable is cytologic examination as a screening technic? And second: Will cytologic examination disclose cancer of the mouth when the lesions are so inconspicuous that neoplasia is not suspected?

Between 1956 and 1959, oral examinations were completed for 16,103 patients at the Veterans Administration Hospital, Brooklyn, N. Y. Every abnormality of the oral or oropharyngeal mucosa was noted and the surface was scraped for cytological study, regardless of how insignificant the lesion, or how obvious the clinical diagnosis, and whether or not biopsy was contemplated.

In these first three years of study, there were 412 patients who presented with 452 abnormalities of the oral mucosa. In 213 cases, the cytology was normal and biopsies were neither recommended nor taken. No conclusions can be drawn from these cases. The remaining 239 oral abnormalities were not only scraped for cytology but were subsequently either excised *in toto* or a specimen of tissue was taken for biopsy. These latter constitute the basis for our findings and conclusions.

It was found that both cytology and biopsy were almost equally reliable, insofar as the diagnosis of cancer of the mouth was concerned.[11, 12] Each method was subject to some degree of error, but their correlation was very high and statistically significant. In this first series of 239 cases there was a 91 per cent agreement on a diagnosis of cancer between cytology and biopsy.

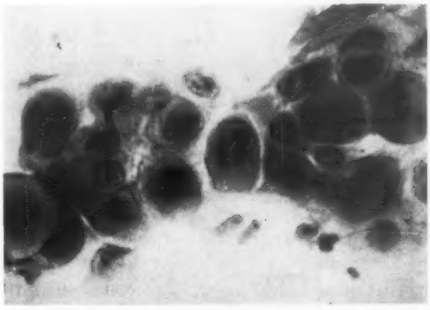

FIG. 1. Scraping from clinically obvious carcinoma of the soft palate (x300, Pap Stain).

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* Chief, Dental Service, Veterans Administration Hospital, Brooklyn, New York.

TABLE 1. *Carcinoma Diagnosis Revealed by Cytology and Confirmed by Biopsy (Pilot Study, August 28, 1956 to October 15, 1959)*

| Cytology findings | Biopsy findings | | Total |
|---|---|---|---|
| | Carcinoma | Not carcinoma | |
| Positive for carcinoma | 131 | 5 | 136 |
| Negative for carcinoma | 15 | 88 | 103 |
| Totals | 146 | 93 | 239 |

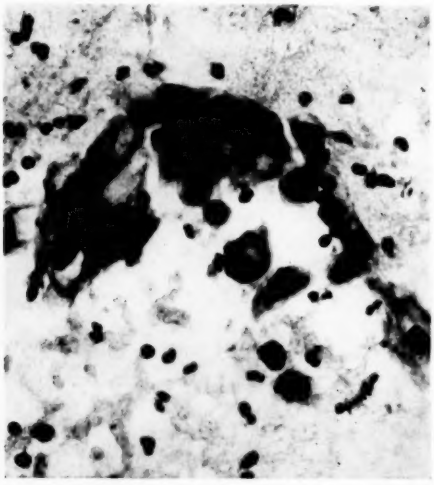

FIG. 2. Scraping from an asymptomatic, irregularly erythematous area, 1 × 2 cm., of the oral mucosa overlying the alveolar process. It was slightly thickened but otherwise normal to palpation. Clinical impression: Nonspecific local erythema. Biopsy: Squamous cell carcinoma (x300, Pap Stain).

There were seven instances where the cytology was suggestive of cancer but multiple biopsies were required to confirm the diagnosis. In four of these, two biopsies were required. In one case, three biopsies were required. In one case, four biopsies were taken, all negative, and the diagnosis was confirmed at postmortem. And in one case, five biopsies were required to confirm the diagnosis.

The oral lesions of 124 patients were clinically suspicious of cancer (Table 2) and were subsequently confirmed by biopsy. However, in seven cases, the clinical appearance of the oral lesion was so innocent that no biopsy was contemplated. It was only because of the strict interpretation of the experimental protocol, namely, that every abnormality recorded was to be included, that a scraping for cytology happened to reveal cancer.

None of these unsuspected cancers of the mouth was larger than 1 cm. in diameter. In three cases, the only indication of abnormality was a granular texture of the sur-

TABLE 2. *Cytology and Biopsy Diagnosis of Carcinoma from 239 Oral Lesions*

| Cytology findings | Biopsy findings | Clinical appearance | Number of cases |
|---|---|---|---|
| Carcinoma | Carcinoma | Suspect | 124 |
| Carcinoma | Carcinoma | Not suspect | 7 |
| Not carcinoma | Not carcinoma | | 88 |
| Not carcinoma | Carcinoma | | 15 |
| Carcinoma | Not carcinoma | | 5 |
| Totals | | | 239 |

face mucosa, and localized erythema. In two cases the mucosa was somewhat thickened and of a somewhat lighter color than the surrounding tissues. In the remaining two cases a thin, white superficial membrane readily removed by scraping was surrounded by an irregular, sharply defined inflammatory outline. All were asymptomatic.

Based upon this pilot study the Veterans Administration is now conducting a Cooperative Study in which the Dental Services of 12 Veterans Administration hospitals are participating. In the first nine months of this cooperative study the cytological specimens of 611 patients have been processed, and 101 of these were verified cancer of the mouth. Ninety-one of these presented some clinical suspicion of cancer of the mouth and were scheduled for biopsy. But there were also ten cases, 10 per cent, which were clinically unsuspected, were *not* scheduled for biopsy and were detected by cytology.

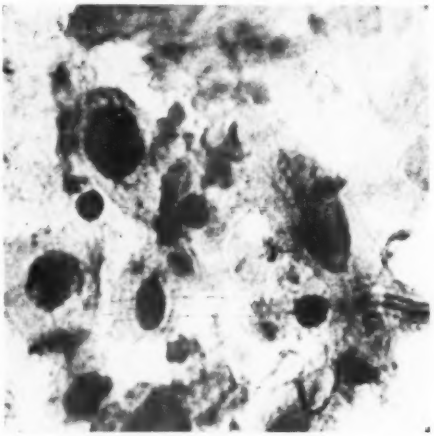

FIG. 3. Scraping from an asymptomatic, irregularly erythematous area, 1 × 1½ cm., of the oral mucosa anterior to the glossopalatine raphae. Normal to palpation. Clinical impression: Non-specific local erythema. Biopsy: Squamous cell carcinoma (x300, Pap Stain).

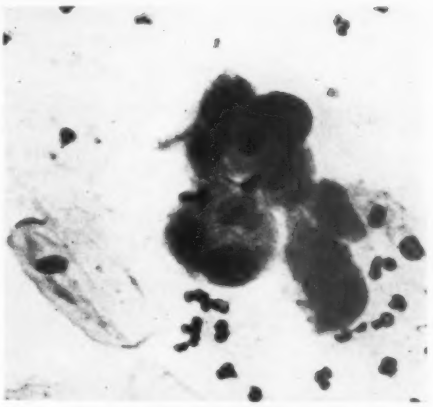

FIG. 4. Scraping from an asymptomatic, 1 × 1 cm. enlargement of the lateral border of the tongue, of rubbery consistency but no visible break in the surface mucosa. Clinical impression: Fibroma. Biopsy: Squamous cell carcinoma (x300, Pap Stain).

## Summary.

It is concluded from these studies that cytologic examination is a reliable screening procedure for the detection of early carcinoma of the mouth; that it can reveal early (intraepithelial) oral cancer when the clinical appearance is so innocent that biopsy may not be warranted; and that cyto-

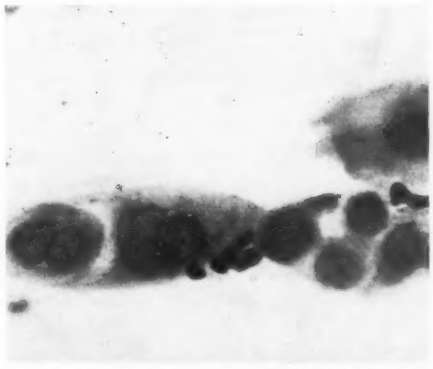

FIG. 5. Scraping from an asymptomatic, 1 × 1 cm., sharply defined, slightly raised, pallid area of the oral mucosa at the junction of the hard palate. Normal to palpation and no break of the surface mucosa. Clinical impression: Early leukoplakia. Biopsy: Intra-epithelial carcinoma (x300, Pap Stain).

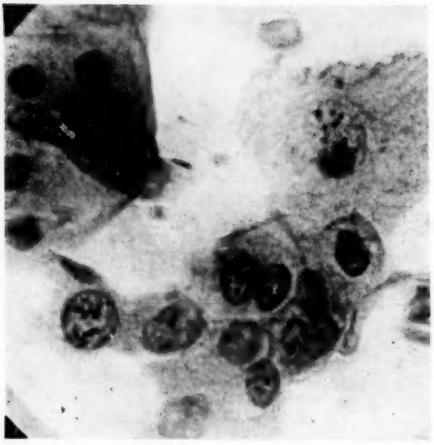

FIG. 6. Scraping from an 0.5 × 0.5 cm. ulcer of the lateral border of the tongue, known to have been present for several months, normal to palpation. Clinical impression: Chronic traumatic ulcer. Biopsy: Intraepithelial carcinoma (x300, Pap Stain).

logic examination supplements biopsy, for it can often provide evidence of cancer when the biopsy specimen is negative.

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# Exfoliative Cytology of the Urinary Tract: Evaluation of the Millipore Technic*

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The purpose of this paper is to evaluate the use of Millipore filters in preparing specimens of urine for cytologic examination.

Early efforts to use exfoliative cytology in the examination of urine resulted in the detection of neoplastic cells, but also yielded 20-30 per cent false negative examinations and a high number of poor preparations. These efforts utilized a variety of technics, all aimed at producing a preparation containing an adequate number of cells suitable for cytologic examination.

Seal,[2] in 1956, first reported on the use of Millipore filters for concentration of cancer cells suspended in large quantities of fluid. Solomen, *et al.*,[3, 4] in 1958, and several other workers since, adapted the filter membrane technic to the examination of urine. Merritt, Henderson and Slate,[1] in 1959, reported the examination of 100 urine specimens from 96 patients using a Millipore technic. There were 11 positive cytologic examinations from eight patients with cancers of the urinary tract. One of six patients with cytologic examination reported "suspicious" had recurrent bladder cancer. The remaining five did not show malignant tumors clinically. Nine preparations were reported as unsatisfactory. There were no reported false negative examinations.

## Materials and Methods

One hundred urine specimens, all obtained during cystoscopy, and chosen at random in the Urology Clinic of St. Vincent Hospital constitute the clinical material for this study. The results of the cystoscopic examination and the biopsy, if one were taken, are compared with the results of the cytologic examination.

The clinical diagnoses are given in Table 1.

All tumors were transitional cell carcinomas. No instance of papilloma was encountered during the course of the study. The diagnoses of the negative group included 45 normal bladders and 36 showing various types of inflammatory lesions.

## Methods

Four-ounce bottles containing 50 ml. of 50 per cent ethyl alcohol were supplied to the Urology Clinic. A quantity of urine, ranging from 2 to 100 ml., was collected directly into the alcohol bottle at the time of cystoscopy.

In the Cytology Department 30 minutes to four hours later, the specimens were filtered through a 47 mm. diameter SM Millipore filter having a pore size of 5 microns ± 1.2 microns. Ten ml. of the urine-alcohol mixture was filtered with the aid of 20 mm. of mercury negative pressure supplied by a water pump.

The precise technic is as follows: Mount the filter in the funnel over the protective

* Presented at the Eighth Annual Meeting of the Inter-Society Cytology Council, 1960, Chicago, Illinois, U.S.A.

TABLE 1. *Clinical Diagnosis of Cystoscopic Examinations*

| | |
|---|---|
| Negative for malignant neoplasm | 81 cases |
| Positive for malignant neoplasm | 19 cases |
| Total | 100 cases |

pad supplied by the manufacturer. Wet the filter with approximately 100 ml. of normal saline. During this period the vacuum can be adjusted to the proper level. Follow the normal saline with the urine-alcohol mixture. The rate and amount of urine which can be filtered varies with the turbidity of the specimen. Filtration is discontinued if air bubbles appear in the column of filtrate coming through the funnel spout.

Follow the urine-alcohol mixture with an additional 100-200 ml. of normal saline to wash down cells on the sides of the funnel and to even the layer of cells on the filter. Place the filter and pad, without drying, in a jar containing 99 per cent isopropyl alcohol for fixation. Filters have remained in this for as long as four days without apparent harm.

Carnoy's solution is also a satisfactory fixative. Ethyl alcohol fixation results in distortion of the filter and produces an uneven field which is very difficult to examine microscopically.

After fixation the filter is attached to a 2″ × 3″ microscope slide with two large uncoated spring-type hair clips. This allows multiple slides to be stained at one time in the usual laboratory staining rack.

Some laboratories have reported difficulty in staining the filters because many commercial stains are made with methyl alcohol which dissolves the filter. This has never been a problem with us because we have always made our own stains, using ethyl alcohol.

After clearing in xylol for about 20 minutes, the filter is mounted on the slide to which it was clipped during staining, using Permount as the mounting medium. More Permount is added on top of the filter and it is coverslipped. Care must be taken during this mounting and coverslipping operation that the filter does not dry out.

TABLE 2. *Cytologic Classification of 100 Urine Specimens Obtained at Cystoscopy*

| | |
|---|---|
| Class I | 61 |
| Class II | 21 |
| Class III | 4 |
| Class IV | 12 |
| Class V | 2 |
| Total | 100 |

TABLE 3. *Cytologic Classification of Urine in 81 Cases Negative for Tumor on Cystoscopic Examination*

| | |
|---|---|
| Class I | 57 |
| Class II | 20 |
| Class III | 1 |
| Class IV | 3 |
| Class V | 0 |
| Total | 81 |

## Results

Cytologic classification of the 100 cases is given in Table 2.

In the group of 81 cases judged negative for tumor on cystoscopic examination, 77 showed correspondingly negative cytologic evaluation. Four cases in this group were classified as suspicious or positive. One of these patients died of cardiac arrest during an operation for bowel cancer. Unfortunately, sections of the bladder were not taken at autopsy. Two other patients had bladder tumors in recent years, and both were treated by partial resection and radon seeds. However, cystoscopy at present is negative for tumor. The fourth patient in this group has urethral stenosis and trabeculation of the bladder. Continued evaluation of these patients is in order.

TABLE 4. *Cytologic Classification of Urine in 19 Cases Positive for Tumor on Cystoscopic Examination*

| | |
|---|---|
| Class I | 4 |
| Class II | 1 |
| Class III | 3 |
| Class IV | 9 |
| Class V | 2 |
| Total | 19 |

In the group of 19 cases judged positive for tumor on cystoscopic examination, 14 showed correspondingly suspicious or positive cytologic findings. There were five false negative cytologic examinations. In three of these, re-examination of the specimens shows malignant cells. In two cases malignant cells cannot be detected. In one of these the diagnosis of recurrent tumor is based on clinical appearance only.

## Discussion

Thus, there was an over-all correspondence between the cystoscopic examination and biopsy with the cytologic classification in 91 per cent of 100 cases. There were four apparent false positive cytologic examinations and five apparent false negative cytologic examinations. All preparations were satisfactory for microscopic examination.

This degree of accuracy will probably be increased as we gain further experience.

## Conclusions

While this is a rather limited series, the following conclusions seem warranted:

1. The Millipore technic as described herein is practical and can be used for the cytologic examination of large numbers of urine specimens.

2. Over-all accuracy of more than 90 per cent can be expected when comparing cytologic and cystoscopic findings.

3. A small number of false positive and false negative interpretations may be expected in the cytologic examination of urine.

4. Based on the results of 100 cases, we are convinced that this is a satisfactory method for the cytologic examination of urine specimens.

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# Cytologic Study of Intracranial Fluids*

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SINCE the impetus to exfoliative cancer cytology was given in the early 1940's, the investigation of fluid derived from the cerebrospinal axis has received scant attention. However, there have been since the beginning of this century some three-score publications which mention specific instances of the finding of cancer cells in cerebrospinal fluid (C.S.F.); these do not take into account tumors of the malignant lymphoma and leukemia group. Most, by far, have reported the finding of tumor cells in C.S.F. obtained by lumbar puncture: some give accounts of the finding of tumor cells in fluids derived directly from the cranial cavity. There are five publications[1-5] which account for many of the reported cases of tumor cells recovered from the intracranial fluids. The number of these cases is 17; the number of specimens involved is 20; 12 of these were C.S.F. derived from lateral ventricles; the remainder were fluid obtained from the cisterna magna, cerebral or pituitary cysts, or a leaking craniotomy wound.

We have been examining intracranial fluids during the last two years with a view to finding out how frequently and how easily tumor cells can be recovered from them, what factors influence such recovery, whether the cells of metastatic intracranial neoplasms can be distinguished from those of gliomas, and what usefulness this line of investigation may hold.

We examined 76 intracranial fluids from 76 patients during a two year period. The neurosurgeons were encouraged to submit to us any intracranial fluid whether or not the patient was suspected of having an intracranial neoplasm. Table 1 shows the type of fluids which we received. The sediments of these fluids were examined by two methods: first, the serum toluidine-blue stained wet-film described by McCormack and colleagues;[6] and second, the Papanicolaou-stained wet-fixed smear. The wet-film method proved to be interesting and useful. It has enabled us to make some rapid diagnoses and also to anticipate the need for a greater number of permanent smears than we usually prepare. Rather than separate the coverslip and the slide and use these for the permanent preparation, we have found it more convenient to discard the wet-film and make permanent smears anew from the sediment.

Figure 1 shows a cell of a metastatic malignant melanoblastoma in ventricular C.S.F. We interpreted this and similar cells as being derived from a malignant epithelial neoplasm; we were unaware of the fact that this patient had a mediastinal mass or that he had had a malignant melanoblastoma removed from the dorsum of his foot 13 years previously. At necropsy the brain was found to be studded by hundreds of black metastases of malignant melanoblastoma. The next case (Fig. 2) shows a cell found in the ventricular C.S.F. of a 29-year-old woman who had been a diagnostic problem

* Presented at the Eighth Annual Meeting of the Inter-Society Cytology Council, 1960, Chicago, Illinois, U.S.A.

This investigation was supported by a grant from the American Cancer Society Institutional Grant 43F.

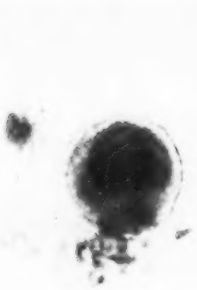

FIG. 1. Ventricular C.S.F. Metastatic melanoblastoma (x1470). FIG. 2. Ventricular C.S.F. Metastatic bronchiolar cell carcinoma (x1470).

for some six months. This was interpreted as adenocarcinoma and came as a great surprise to the clinicians. At necropsy she was found to have thousands of minute nodules of bronchiolar-cell carcinoma in the lungs as well as meningeal carcinomatosis. The third case is similar; a 44-year-old man who had been a diagnostic problem for ten months. This is one of the cells (Fig. 3) which was recovered from the ventricular C.S.F. This again was interpreted as adenocarcinoma, and he too was shown at necropsy to have bronchiolar-cell carcinoma involving the lungs, with metastases to the brain. Note in these three illustrations the clear-cut boundaries of the cells and the abundance of cytoplasm.

Figure 4 shows the cells of a grade III astrocytoma recovered from ventricular C.S.F. Note that the cell is dominated by a pair of nuclei and that the cell boundaries are "woolly" and the cytoplasm scanty. Case 5 shows cells of a pinealoma which were recovered from ventricular C.S.F. (Fig. 5). Here again they are dominated by nuclei and the cytoplasm is indistinct; there is also a number of necrotic cells in this field. Figure 6 shows cells of an ependymoma found in the C.S.F. of the cisterna magna. Because of the shape and arrange-

FIG. 3. Ventricular C.S.F. Metastatic bronchiolar cell carcinoma. Stained wet film (x1200).

FIG. 4. Ventricular C.S.F. Astrocytoma, grade III (x1470).

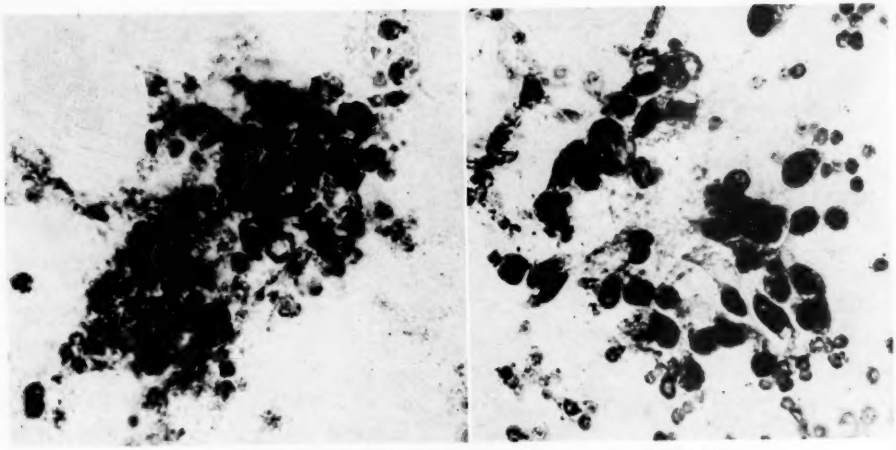

FIG. 5. (Left) Ventricular C.S.F. Pinealoma (x690). FIG. 6. (Right) Cisterna magna C.S.F. Ependymoma (x585).

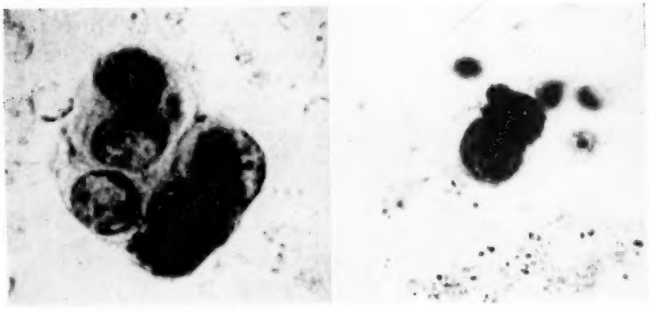

FIG. 7. Pituitary cyst aspirate. Chromophobe adenoma (x1470). FIG. 8. Cerebral cyst aspirate. Astrocytoma, grade II (x1250).

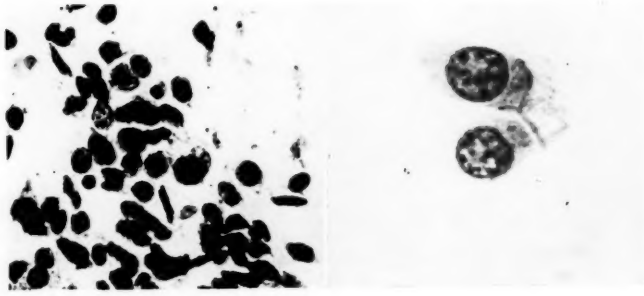

FIG. 9. Cerebral cyst aspirate. Oligodendroglioma (x585). FIG. 10. Porencephalic cyst aspirate. Ciliated columnar cells (x1470).

TABLE 1. *Sources of Fluids*

| | |
|---|---|
| Lateral ventricles | 66 |
| Cerebral cysts | 6 |
| Cisterna magna | 2 |
| Cerebellar cyst | 1 |
| Pituitary cyst | 1 |

ment of the nuclei, and the long cytoplasmic processes, we suggested that these were cells from an ependymoma or medulloblastoma.

Cells derived from a chromophobe adenoma of the pituitary body are shown in Figure 7. These cells were recovered from fluid aspirated from what was thought to be an internal carotid arterial aneurysm. We reported these as atypical epithelial cells, much to the astonishment of the clinicians. The discovery of these cells, and the fact that the patient did not improve after the "aneurysm" had been clipped, caused a revision in diagnosis and re-operation. At the second operation a cystic pituitary chromophobe adenoma was partly removed.

Case 8 illustrates the cells aspirated from a cystic grade II astrocytoma (Fig. 8). Note that the cell has very little cytoplasm and the boundary is indistinct. The cells of a cystic oligodendroglioma are shown in Figure 9. Their nuclei are more hyperchromatic than those of the other gliomas that have been shown, but again the cytoplasm is almost nonexistent.

Last — a cytologic curiosity: Figure 10 shows the cells recovered from a porencephalic cyst in an 18-year-old young man. Our first reaction was that these were contaminants from bronchial secretions and so we took all the necessary precautions to exclude this. They appear to be immature ependymal lining cells, which in infancy may be ciliated and occasionally they persist as such until adulthood.

TABLE 3. *Gliomas and Metastases: Cell Recovery*

| | Number | Tumor cells recovered |
|---|---|---|
| Gliomas | 26 | 5 |
| Metastases | 8 | 3 |
| | 34 | 8 |

Table 2 shows the number of intracranial neoplasms which were involved, their types, and the number of specimens from which tumor cells were recovered. Only one benign neoplasm, that of the pituitary adenoma, gave tumor cells in fluids. The malignant neoplasms gave a higher proportion of cell recovery. Table 3 shows the division of the malignant neoplasms into gliomas and metastases. It will be seen that a higher proportion of the metastatic neoplasms gave recoverable tumor cells than did the gliomas.

In the case of ventricular C.S.F., recovery of tumor cells will depend on whether the neoplasm encroaches upon the wall of the ventricle or not. That this is not the whole story is shown in Table 4. You will note that although eight gliomas involved the walls of the lateral ventricles, in only two were tumor cells recovered; whereas in the case of five metastatic epithelial neoplasms, tumor cells were recovered in three. Probably the recovery of tumor cells depends

TABLE 2. *Benign and Malignant Neoplasms: Cell Recovery*

| | Intracranial neoplasm | Tumor cells recovered |
|---|---|---|
| Benign | 12 | 1 |
| Malignant | 34 | 8 |
| | 46 | 9 |

TABLE 4. *Tumors Involving Lateral Ventricles*

| | Types | Tumor cells recovered |
|---|---|---|
| Gliomas | 8 | 2 |
| Metastases | 5 | 3 |
| | 13 | 5 |

upon the ability of the tumors to exfoliate cells. Presumably the epithelial neoplasms possess this inherent property to a greater degree than do the gliomas and the benign neoplasms.

This study has shown a distinct difference between the exfoliated cells of the epithelial neoplasms and the gliomas. The former have well-defined cell boundaries and abundant cytoplasm; the latter have "woolly" margins and scanty cytoplasm. Often the exfoliated cells of astrocytomas of all grades have consisted of a pair of overlapping round to kidney-shaped nuclei with scanty cytoplasm.

The detection of cells of gliomas will seldom be of great value to the clinician. In most gliomas, the neoplasm has been satisfactorily located before operation and frozen sections during operation add the confirming histologic evidence. The finding of exfoliated glioma cells may, on occasion, be useful in cases of suspected recurrence of glioma when a decision as to whether or not to re-operate has to be made.

Most usefulness for the procedure will be obtained from examination of fluids in cases of suspected intracranial neoplasm when there are no definite localizing features, and especially when the possibility of metastatic intracranial neoplasm is entertained; this was our experience in Case 1. The finding of metastatic tumor cells in intracranial fluid may come as a complete surprise as in Cases 2 and 3. Such a finding may refocus attention on other systems of the body and enable a primary neoplasm to be identified and a definite diagnosis to be made, as in Case 2. There is also a place for the examination of intracranial fluids from lesions which cannot be resected or biopsied, such as the pinealomas and other deeply-situated gliomas.

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# A Method for Demonstrating Malignant Cells in the Blood

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A GREAT number of problems of clinical importance can be approached by employing technics which isolate cancer cells from the peripheral blood.[2, 3, 7] The implications of such studies are too obvious to require emphasis. Personal experience over the past eighteen months, however, has convinced us that the technical development generally lags behind attempted applications. This paper concerns itself with some technical improvements and modifications of existing technics we are now employing.

One of the main difficulties encountered in blood cytology lies in the fact that one is dealing with a very great number of cells. As an example, a Millipore filter may contain as many as ten to fifteen million white cells. Numerous artefacts are created due to this enormous concentration of cells on an area of about 10 square centimeters. Naturally this may produce confusing cell clumps and thus interfere with the screening of the slides. Furthermore, the time involved in the examination of such filters is relatively long. For these reasons and, with a background of personal experience with at least three of the several existing methods, we have tried to improve some aspects of the technics commonly in use today.[2, 4, 5, 11] Generally, one attempts to hemolyze the red cells, be it with plain water—a process that takes a relatively long time—or be it with acetic acid, which produces damage to the white cells also. Another approach is the differential centrifugation whereby the cellular elements are separated on the basis of differences in their specific gravity.[8, 9, 11] Still another approach is the use of enzymes, such as streptolysin O —an enzyme which also destroys many of the polymorphonuclear leukocytes. We employ saponin for the purpose of inducing hemolysis. Depending on its concentration, however, saponin, too, is capable of destroying the polymorphonuclear cells as well as the lymphocytes. We use calcium gluconate to inhibit this effect of saponin on the white blood cells. The assumption is made that saponin, when used in concentrations which do not affect the white blood cells, is also ineffective against cancer cells. Briefly, we are now using the following technic:

A solution, hereafter referred to as Tween-80 solution, is used for the purpose of suspending the cells. It contains 0.5 per cent of polyvinylpyrrolidone (Plasdone C, K—30) —a polymer which apparently coats the individual cellular elements. Dissolved in the same solution are: WR—1339 (1 per cent) —a non-ionic emulsifier; Sequestrene NA2 (.1 per cent)—a widely used chelating agent; and Tween-80 (.3 per cent) —similarly a non-ionic emulsifier. We employ two different fixatives: Fixative I consists of seven parts of buffered formalin, three parts of 95 per cent ethyl alcohol, and enough

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This investigation was carried out during the tenure of a Postdoctoral Fellowship from the National Cancer Institute United States Public Health Service. (B. N.)

glacial acetic acid to give a *p*H of 7. Fixative II (modified Carnoy fixative) contains seven parts of 95 per cent alcohol, two and one-half parts of chloroform and one-half part of glacial acetic acid.[1] Ten milliliters of blood is first mixed with the solution containing the different emulsifiers (Tween-80 solution) and centrifuged at 1,700 rpm. for ten minutes. Following aspiration of the supernatant the remaining cellular elements are resuspended in Tween-80 solution. To this we add 2 ml. of 1 per cent aqueous saponin solution and, after one minute of exposure, the action of saponin is arrested by adding 3 ml. of a 10 per cent calcium gluconate solution. Following another centrifugation at 1,300 rpm. for ten minutes, the supernatant fluid is removed and the pellet is resuspended in Fixative I. After ten more minutes the material is filtered through five Millipore filters which are then fixed with Fixative II for ten minutes. This step is followed by placing the filters into suitable plastic holders and carrying them through the Papanicolaou stains.[6] Finally the filters are mounted on 3 × 2 inch slides, using Permount, and 49 × 49 mm. cover slips.

## Discussion

One of the advantages of this technic is the morphologic preservation of the cells due to the fact that we are dealing with fixed cells and the pressure during filtration is thus of no particular importance. The use of calcium gluconate controls the action of saponin, in fact, it is possible to produce a differential destruction of the white blood cells by selecting the proper concentration of saponin, and the proper exposure of the cells to its action. Another advantage appears to lie in the fact that the technic is quantitative in the sense that all the preserved cellular elements can be recovered. Examination of the supernatant fluid has shown that a very insignificant number of cells may be lost. Furthermore, by adding a counted number of identifiable cells to a blood specimen and processing it by this method, one can evaluate this aspect of the technic. We have used several different types of cells, such as tissue culture cells from dog kidney, monkey kidney, synovial cells, HeLa cells, and carcinoma cells obtained from pleural effusions. Still another approach to the evaluation of the quantitativeness of this method lies in the comparison with technics which are purported to be quantitative.[5] Such experiments have convinced us that it is possible to recover almost all added cells. A further advantage may be ascribed to the fact that the materials used are rather inexpensive and readily available. The time of processing is relatively short – one-half hour if one wishes to stop prior to filtration, or approximately one hour for the complete processing of the specimen. In the fixed state the cells may be kept for long periods of time. Comparative studies with follow up specimens may thus be performed. The time necessary for screening varies with the number of slides. Since only a few simple steps are involved, the method is easy to learn and does not require any specially trained personnel other than for screening. The number of artefacts is rather low. Finally, it is possible to process simultaneously a relatively large number of specimens, the limit being the number of places in the centrifuge head.

## Summary

A simple, inexpensive and quantitative method for the isolation of malignant cells from the peripheral blood is outlined. The difficulties commonly encountered, and the relative advantages of this modification of existing technics are discussed.

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# Cytotechnology Section of the Inter-Society Cytology Council

### Slide Exchange Program

The attendants of the meeting of Cytotechnologists at the Eighth Annual Meeting of the I.S.C.C. in Chicago last September 23, 1960 will remember one of the ideas posed for their consideration was that of a Slide Exchange Program.

As outlined in the mimeographed letter (from Mrs. Grace Durfee, Memorial Center for Cancer and Allied Diseases, New York 21, New York) which the attendants of the meeting received, we are interested in establishing a Slide Exchange Program. The purposes of which are to make available slides for Registry Examination preparation and to create study sets for those who wish to temporarily supplement their own teaching slides.

The following questions are extended:

1. Are you interested in such a program?
2. Would you be willing to contribute slides to such a program?
3. What suggestions do you have regarding such a program?

Some communications regarding a slide exchange program were received here, so there is some interest. Now, it is necessary to determine the extent of interest and whether enough slides could be obtained to establish a worthwhile exchange.

The readers of this section are requested to write to Mr. Marvin W. Stafford, C.T. (ASCP), 3426 Conrad Street, Philadelphia 29, Pennsylvania, so that more definite plans may be made.

### Consultant's Corner

A future issue will see the inauguration of the "Consultant's Corner." The first Consultant will be Dr. James W. Reagan, Professor of Pathology, Western Reserve University, Cleveland, Ohio. Any questions that the readers would care to ask Dr. Reagan should be submitted to Mr. Stafford at the above address, and as many as possible will be answered in the next Cytotechnology Section.

This should be one of the bright new features of the cytotechnology section. Send along your questions, and we shall all benefit from the vast experience of our consultants.

### Tips on Technics

A technic for the handling of cell block material was reported by Dr. John W. Eiman, Department of Pathology, Abington Memorial Hospital, Abington, Pennsylvania in his paper presented at the Annual Meeting of the American College of Obstetricians and Gynecologists in Cincinnati, Ohio, 1960. The title of the paper was "Spiral Curet Cervical Biopsy as an Office Screening Procedure:"

The specimen is placed in a polyethylene bottle cap (non-opaque, snap-on type, commonly stocked in the pharmacy). The cap is covered with a disc of filter paper, then placed in the standard tissue receptacle (Casette). This method keeps the material concentrated and cuts down on the loss of material in the Auto Technicon baths.

### Feature Laboratory: University of Illinois

The Feature Laboratory for this Cytotechnology Section is the Cytology Laboratory of the University of Illinois, College of Medicine, Chicago, Illinois.

(continued on page 210)

# The Cytology of Degenerative and Inflammatory Lesions*

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THE ENUNCIATION of the cell theory by Schwann in 1847 marked the birth of cytology as a discipline. Eleven years later Virchow[38] revolutionized pathology with publication of his treatise *Cellular-pathologie* by considering the cell both as the ultimate morphologic element of which living tissues are composed, and the fundamental unit affected by disease. Thus, it is not surprising that today we have returned to the description and study of disease at the cellular level[2, 9, 28] due to major advances in cytology and to the development and application of the electron microscope, cytochemistry and autoradiography. Cytology will be used here not only in the sense of diagnostic cytology but as the detailed study of the cell and its reactions to adverse environmental conditions. The present communication will deal with some recent developments in cytology and biochemistry as they apply to cellular mechanisms encountered in various degenerative and inflammatory lesions in an attempt to broaden our understanding and interpretation of cellular reactions seen in the diagnostic cytology laboratory.

## Degenerative Lesions

In degeneration, alterations in cellular architecture occur both in the cytoplasm and nucleus. The earliest changes become evident in the cytoplasm which assumes a granular appearance that later progresses to a foamy and finally a vacuolar one.[5, 29, 39] Electron microscopic studies have shown that these changes are due mainly to mitochondrial swelling.[8] The usually rod-shaped organelles in which many of the oxidative enzymes are localized and which are responsible for the majority of oxidative metabolism in the cell respond to damage by swelling and finally assume large globose shapes. This leads to major disturbances in oxidative cell metabolism with losses of cell function such as absorption, secretion and synthesis.[3, 17] This phenomenon is observed under the influence of widely differing conditions and agents such as anoxia,[19] starvation,[31] major disturbances in the osmotic pressure of intracellular and extracellular fluids,[27] noxious compounds such as heavy metal salts,[35] bacterial toxins,[10] and lastly, high concentrations of hormones and vitamins such as thyroxine[14] and vitamin D.[32] In populations of basal cells obtained by biopsy, scraping or exfoliation, many examples of vacuolar degeneration are encountered. These are most numerous in cells obtained by vaginal aspiration since many of these have been desquamated for considerable time and have degenerated.

Recent experimental evidence suggests that the highly organized structure of the basophilic cytoplasm, the endoplasmic reticulum, may be damaged even quicker than mitochondria.[24] This could result in disturbances in protein metabolism and synthesis; corroboration and amplification of these findings are awaited with great interest.

A third cytoplasmic manifestation of cell damage is the abnormal storage of certain substances and retention of abnormal substances. Some of these substances are intimately associated with the mitochondria, with a smaller organelle called the lysosome,

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† Senior Research Fellow (SF-104) from the National Institutes of Health.

or with the basophilic cytoplasm. In some instances material may simply lie within various intra and intercellular spaces.

The accumulation of fat and fatty substances in the cytoplasm of damaged cells is a well known pathologic phenomenon.[6] Electron microscopic and biochemical evidence show that the accumulation occurs within mitochondria.[24] However, despite the application of more sophisticated methods of study the question that plagues the general pathologist is still unsolved; namely, is the accumulation of fat the result of intramitochondrial lipids which have been altered, or merely the reflection of the inability of these organelles to metabolize fatty substances transported to them from fat depots? Thus, the difficult differentiation between fatty degeneration and fatty infiltration remains.

The intramitochondrial accumulation of protein has been observed in the proximal convoluted tubules of the kidney during protein absorption.[30] The mitochondria swell and become filled with a dense substance, these fuse together to form large granules which some workers feel correspond to eosinophilic cytoplasmic droplets seen in tissue sections in hyaline droplet degeneration.[26]

The accumulation of keratohyaline granules which coalesce into 1-2$\mu$ bodies was first described in degenerating squamous epithelium in vitamin A deficiency. These bodies are often encountered in exfoliated squamous cells in vaginal smears in various types of epithelial dysplasia. They exhibit an intensely basophilic staining reaction, and histochemically display a strong positive reaction for disulfide groups, one of the chief constituents of keratin. Studies on the ultrafine structure of these bodies show that they may represent altered mitochondria in which an electron dense structureless material is deposited.[33]

Recently both biochemical and morphologic data[7, 22] have pointed to the existence of still another organelle called the lysosome, possessing a densely osmiophilic body smaller than a mitochondrion in which are localized a series of hydrolases, namely, acid phosphatase, acid ribonuclease, acid deoxyribonuclease, cathepsin, $\beta$-glucuronidase and aryl-sulphatase. These enzymes are concerned with the enzymatic hydrolysis of complex substances and as we shall see later play an important role in the cellular response in inflammation. In certain degenerative lesions in liver cells, lysosomes have been implicated on the basis of both histochemical and electron microscopic evidence.[8, 23] In a liver containing large amounts of lipofuscin pigment, enlarged cytoplasmic granules resembling lysosomes and containing a moderately dense homogeneous material and a peripheral rim of ferritin-like granules were observed. Histochemically the large granules exhibited an intense acid phosphatase activity and were distributed in the pericanalicular regions of the liver cords. They are thought to correspond to lysosomes containing lipofuscin. In a case of idiopathic chronic jaundice of the Dubin-Johnson type similar lysosome-like, pigment-containing granules were observed. In a case of obstructive jaundice, bile pigment appeared localized within small dense bodies exhibiting high acid phosphatase activity and resembling lysosomes, some of which were present in distended biliary canaliculi. These data suggest that lysosomes may play a role in the intracellular transport of bile and iron and may be modified by deposition of pigment to the lipofuscin granules.

Although, in some instances these data are supported by, and are consonant with current biochemical thinking, they must be interpreted with some caution. It may be that the presence of a substance within an organelle does not necessarily mean that the substance has been synthesized there, nor that it is metabolized by the organelle.

The possibility remains that intraorganellar accumulation of a substance may be purely accidental.

Nuclear changes lag considerably behind cytoplasmic ones. It has been shown by cytospectrophotometric studies of various nuclear proteins that in cellular degeneration and even in necrosis depolymerization and digestion of nuclear proteins is a relatively slow process.[15] Pyknosis, karyorrhexis and karyolysis occur long after cytoplasmic dissolution and nuclei are often seen in exfoliated material as isolated nuclei devoid of surrounding cytoplasm, the so-called "naked nuclei." The slow lysis of nuclear material observed by the cytologist is supported by the biochemical finding that action of deoxyribonuclease and ribonuclease results in formation of cyclic phosphates which are hydrolyzed slowly.[18]

### Inflammatory Lesions

Inflammatory lesions are characterized primarily by the type and number of cells constituting the exudate. In acute inflammatory lesions polymorphonuclear leukocytes predominate. In cytologic smears groups of leukocytes often surround necrotic cells[36] aiding in their digestion by releasing various hydrolytic enzymes. Acid phosphatase, $\beta$-glucuronidase and cathepsins have been studied histochemically in smears from a variety of acute inflammatory lesions ranging from cervicitis to empyema. In each instance polymorphonuclear leukocytes exhibited high enzyme activity.

Exudates in chronic inflammatory states may contain lymphocytes, monocytes, macrophages and plasma cells. The following discussion will briefly deal with the structure and possible function of several of these cells in the chronic inflammatory response as suggested by recent studies. The role of the lymphocyte in inflammation is at present obscure. Although it has been suggested that its primary function is that of antibody synthesis,[13] there is little experimental evidence to support this. It may be that the lymphocytes carry antibodies synthesized elsewhere in the reticuloendothelial system. Recent immunochemical studies[4] have shown that the majority if not all antibody synthesis resides in the plasma cell. These findings are supported by the morphology of the plasma cell. Under the light microscope plasma cells are characterized by an intensely basophilic cytoplasm which under the electron microscope is resolved into a highly developed ergastoplasm consisting of numerous cisternae lined by membranes and surrounded by numerous ribonucleoprotein granules. This represents one of the major systems responsible for protein synthesis. These findings point to the function of these cells as being that of antibody protein synthesis. the humoral defense mechanism which protects the cell from the cytotoxic effects of foreign proteins.

Macrophages also exhibit intensely active hydrolytic enzyme activity, especially those in the process of phagocytosis. Histochemically intense intracellular catheptic activity was observed in and around the ingested material. The marked catheptic activity exhibited by macrophages following short incubation not exceeding 15 minutes contrasted sharply with the weak activity displayed by neoplastic cells. This enzymatic difference between inflammatory and neoplastic cells is not as evident following incubation periods of from 30 to 40 minutes, due to a progressive increase in the intensity of the tumor cell reaction. The possibility of utilizing this method as a diagnostic aid in differentiating stimulated mesothelial cells from neoplastic ones is being presently investigated in our laboratory. In cases of pulmonary tubérculosis foreign body giant cells both in pleural fluid and in pulmonary tissue sections exhibited identical enzyme activities. Electron microscopic studies have shed much light on the process of phagocytosis and intracellular digestion.

Evidence has now been accumulated to show that the process of phagocytosis is

mediated by the moving or flowing of the plasma membrane by which extracellular materials may gain entrance into the cytoplasm.[1, 25] This process is identical to that observed in tissue culture in which the extracellular fluid is imbibed by the cell (pinocytosis) by means of numerous small vesicles formed by an infolding of the plasma membrane which is subsequently pinched off to form isolated intracellular vesicles (caveola intracellularis).[11, 12, 16] The ingested material is then progressively digested by the release of hydrolytic enzymes residing in the lysosomes. There is additional evidence that suggests the existence of an intracellular transport system consisting of pinocytotic vesicles communicating with the cisternae of the ergastoplasm and the nuclear pores.[34] Thus, it is probable that extracellular material may be incorporated into the cell, hydrolyzed enzymatically, following which the products of hydrolysis such as amino acids, simple peptides and low molecular weight carbohydrates are transported to the ergastoplasm, nucleus and mitochondria to be used for energy or synthetic metabolism.

The nuclear and cytoplasmic alterations in various inflammatory states are identical to those described earlier for degenerative lesions with one notable exception, that of the intranuclear and intracytoplasmic inclusion bodies observed in cells infected by viruses.[37] Animal viruses have been found to be moderately complex chemically consisting of either ribonucleic or deoxyribonucleic acid in various combinations with carbohydrates, proteins and lipids. This is supported by the great variability observed in the tinctorial staining and cytochemistry of various viral inclusion bodies. The chemical make-up appears to vary with each virus. This is in contrast to plant viruses which are less complex chemically consisting primarily of ribonucleic acid. Electron microscopic studies of inclusion bodies have resolved these basophilic or eosinophilic round bodies into numerous osmiophilic particles of millimicron size organized into tightly packed crystalline type structures.[20, 21] The individual particles appear to consist in many instances of a dense central core and possess a center to center spacing which is repeated throughout the crystal giving it a geometric "honey comb" appearance. It is evident even from this brief discussion that the biochemical and biophysical approach to cellular pathology has given and will give new and dynamic insights on the structural and functional nature of disease.

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(continued from page 205)

**Stella Nanos, C.T. (ASCP),** Supervisory Cytotechnologist, is the reporter:

The Cytology service of the Department of Pathology, University of Illinois, College of Medicine was opened by Dr. Elizabeth A. McGrew, then Assistant Professor of Pathology with four months training under Dr. George N. Papanicolaou at Cornell University in New York. Serving the Research and Educational Hospitals and Clinics, the laboratory now processes 6,000 specimens annually of which approximately 90 per cent are from the female genital tract. Specimens from outside this institution are accepted only on a consultation basis or for special studies.

The basic staff consists of two registered cytotechnologists, Mrs. Jadvyga Maldeikis and the author, who are responsible for the processing and screening of all routine material. In addition, they assist and advise in the research program, supervising and training additional research laboratory personnel.

The slides are filed so that they are readily available for review and teaching of technologists, residents and fellows. An edge-punched card file permits statistical data and clinical correlation as well as specific cytologic and histologic conditions to be extracted with ease.

Entirely negative routine gynecologic specimens are reported without review of the slides by the pathologists, but descriptive evaluations of all other specimens are made for their subsequent teaching value.

A considerable part of the work of the laboratory is investigative. A flotation method for the separation of cell types and study of their physical properties was developed and is utilized in the preparation of most pleural and peritoneal fluids. This principle was later applied to the isolation of tumor cells from blood. Numerous special stains have been adapted for the identification of special cellular features in cytologic preparations of body fluids, and smears and tissues for nuclear sex determination have been processed in almost 400 patients.

In cooperation with various members of the Medical and Dental faculties, specific cytologic studies have been carried out. These have been concerned with carcinoma of the oral cavity, inflammatory changes in the oral mucosa, cerebrospinal fluid and radiation changes in various tissues and in malignant ascites following radioactive gold. Vascular perfusion specimens and smears from various sites from carcinomas of the colon and lung have been searched for tumor cells, to assist in improving surgical technics. Major research projects in this laboratory have been concerned with carcinogenesis and dysplasia in the mouse and human cervix and with the hematogenous spread of tumor cells in animals.

A weekly meeting of the laboratory staff includes discussion of slides previously circulated and usually a symposium on a condition or system related to cytology. Cytotechnologists from other hospitals attend and each participant reports on a particular aspect of the topic assigned. Recent topics included "Effusions," "Mechanisms of Metastasis," the "Urinary System" and the "Reticulonendothelial System."

The laboratory is under the general guidance of Dr. McGrew who frequently participates in weekly laboratory meetings. However, other members of the Pathology Department share in interpretation of cytologic slides and are available for discussion and consultation. The situation of the laboratory in a large pathology department is very favorable. There is a large amount of interesting clinical material, and a variety of stimulating problems are created by close contact with major research projects.

# Possible Mechanisms for Nuclear-Nucleolar Changes in Early Cervical Carcinoma*

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EARLY cervical carcinomas offer favorable opportunities to study nucleolar physiology. Since exfoliated cells reflect to some degree functional intranuclear changes, it is of importance to know how nucleolar contributions are related to cytoplasmic growth. It is of nearly equal importance to know how far the dehydrative and biochemical changes resulting from exfoliation differ from the basal layer and possible invasive cell cytology.

It has been useful to make detailed histochemical comparisons of human tumors with spontaneous and experimentally transmitted renal cancers of the frog.[3] By study of the much larger nucleus of the frog cell as an exact frame of reference, many heretofore confusing details of human malignant nuclei become orderly and understandable. This paper reports an intensive cytochemical investigation of four clinically and histologically proved, extremely early cervical carcinomas, compared with five similar biopsies taken from normal patients under same conditions and fixed within 120 seconds in modified Tellyesniczky's fluid (70 per cent ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part). Our conclusions have been extended and checked on routinely prepared specimens in pathology collections including ovarian carcinomas, renal adenocarcinomas and malignant melanoma. They also derive from study of various nonmalignant hypertrophies, such as that following unilateral nephrectomy. The amphibian material consisted of some 55 spontaneous renal adenocarcinomas and 22 filtrate-induced malignancies from 105 injected animals that showed graded responses. Most of the tumors were investigated in parallel long-term tissue cultures. We have been fortunate in having an additional 120 spontaneous and induced tumors from Dr. Lucké's original collection.

Histochemical technics, such as the Feulgen reaction and Methyl green-Pyronin Y staining showed in each of the early tumors, as well as in more developed ones, in every nucleus a hyperactive DNA-nucleolar capsule surrounding an enlarged colloidal nucleolar core consisting of RNA and protein. There was no evidence of nucleolar lipid. It has been our experience that the same qualitative relationships hold for all cells, human or animal, normal or malignant. However, it may be stated as a rule that in malignant cells nucleolar RNA is quantitatively greater. Attention is directed to the parallel observations of Drs. H. C. Taylor, Jr. and Margaret Long[7] who noted up to 70 times greater RNA content of Grade III ovarian and endometrial neoplastic nucleolar cores as compared to Grade I. Both in their material and in our own, and in those of others, all stages of extrusion of nucleolar material through the nuclear membrane could be demonstrated. Comparable observations have been reported in cervical tumors by Boschann.[1] Massive extrusions appeared limited to the highest malignancies both in frog and human material. It is worthy of note that

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in two of our cases, and in two other exceptionally malignant instances, the amount of RNA-material so excreted could exceed in many cells more than twice the volume of the corresponding nucleus. Consequently it must be concluded that the cytoplasmic inclusion bodies, rich in RNA, may accumulate before being diffused into the cytoplasm to take part in protein synthesis. In all tumor tissue studied all degrees of enlargement and bursting of the nucleolus were observed and the repetitive nature of the process confirmed. In non-malignancies, but where high rates of mitosis were seen, an intermediate degree of nucleolar hyperactivity was more common.[4] Lucké first noted intranuclear inclusion bodies in amphibian adenocarcinoma, but did not realize their relationships nor their method of formation.

It does not appear to be well known that relatively large objects and masses can traverse the nuclear membrane without injury to the cell. For example, a microneedle can be inserted into a nucleus without damage. In each cell of our early carcinoma cases where Cowdry Type A intranuclear inclusions could be identified, they were found attached to or escaping from a DNA-nucleolar capsule. Numerous instances were demonstrable where the Type A material (RNA) was traversing the nuclear membrane. Frequently this substance in its new location in the cytoplasm was found connected by strands through the membrane to the point of origin. Extrusion has been frequently observed in tissue cultures. There can be no longer any doubt that cytoplasmic RNA is expelled repetitively from the nucleus. Our evidence is accumulating that the rates and mass vary directly with the degree of cytoplasia. This was apparent where invasive cells were so highly crowded and packed together that nucleolar growth and output were hampered by lack of available substrate and intense physiological competition. However, a few micra distant where the cells had opportunity to fan out and invade lymphatic spaces, the nucleolar hyperactivity resumed to genetically determined speed.

Similar to conditions in amphibia, a second important type of nucleolar change distinguishes early cervical carcinoma cells from normal ones. This difference consists in occurrence of densely packed DNA-granules around the nucleolar capsule. Usually they attach to it by threads resembling chromonemata. A designation of *nucleolar associated* chromatin is, therefore, only partly correct. Repetition of technics of Page, Regan, and McCarty[6] using both silver stains and the Feulgen reaction showed that argentophil perinucleolar granules described in 1938 by these authors are actually DNA-particles. It should be recalled that these observers related such granules to degree of malignancy in a wide selection to human tumor patients then at the Mayo clinics, but that they were unable to assess or comprehend their function. In the frog Duryee[2] identified the perinucleolar particles as DNA-replicates of the nucleolar organizer. It became evident that DNA from a tumor could multiply at a recognized gene locus and that it could escape from the cell. Further evidence has made clear the fact that an infective type of DNA exists specifically for the frog kidney, which like a virus, replicates only in living cells, is composed of DNA, and affects a single gene function. It is perhaps too early to draw conclusions for human tumors generally. We simply report here that in each of the cervical malignancies, DNA-perinucleolar granules were prominent in the vast majority of cells, and that normal counterparts were free of such accumulations. In one normal case, clinically reported as squamous metaplasia, an interesting intergradation existed, but this should not be stressed until more cases are studied. In this report, as in our 1960

joint paper, we offer no implication of any human tumor virus. Accurate comparisons of animal with human tissues are, however, of present value. It is simply of interest to note that in favorable locations "new" DNA-granules were observed, not scattered at random, but were concentrated near or on the chromosomal attachment point to the nucleolus, or escaping into the cytoplasm.

Our observations have led us to certain definite conclusions as to the nature and origin of the malignant process in lower animals. It should be cautioned that the word or term *virus* can often be replaced with the term "infective DNA." No evidence that we know now definitely contradicts the concept that cancer is essentially a disease of the nucleolar machinery. This point of view fits well with a vast amount of genetic data, with widely accepted biochemical views, on RNA control of protein synthesis, and with much of the ever growing experimental evidence. These observations substantiate and extend Furth's[5] cytogenetic theory of carcinogenesis. The fact that new DNA can infect a cell, can replicate on a host gene template, and thus stimulate a normal function to a malignant degree should be kept in mind while observing unexplained human cytological events. The interpretations may be of value in explaining commonly noted nucleolar and nuclear patterns in exfoliated cells. An improved rationale can be an aid to diagnosis.

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# A Comparison of the Vaginal Aspiration and Cervical Scraping Technics in the Screening Process for Uterine Cancer*

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SEVERAL different cytologic technics are available by which cellular evidence of uterine cancer can be detected, and it is imperative that the relative efficacy of each be tested by many investigators and on a large number of women.[1-3, 7, 9]

One, of the objectives of our Cancer Survey Project was the comparative evaluation of the vaginal aspirate and the cervical scraping technics as aids in the detection of uterine neoplastic lesions.[8, 12] Another objective was to rescreen as many women as possible for three consecutive years in order to determine the efficiency of the screening procedures and to determine the number of cases developing during the year.[4-6, 10, 11] Over a four-year period 200,000 separate cytologic examinations were performed on 113,000 women, which represent almost 50 per cent of the adult female population of the community. Sixty thousand of these included both a vaginal aspirate and a cervical scraping, the remainder only a vaginal aspirate. Seventy-three per cent of the cell studies were submitted by private physicians in Columbus, Ohio. The remainder came from the outpatient clinics of the Ohio State University Health Center and from special clinics organized in various industries and other areas specifically for the routine screening of asymptomatic adult women.

The initial screening of the slides was performed by cytotechnologists and reported according to Papanicolaou's classification. All slides other than those that were completely negative were rescreened by a reviewing cytotechnologist and the chief cytologist, and finally were examined by one to three pathologists. In cases where two or more types of cytologic specimens were submitted, each was independently evaluated and classified. Biopsy was requested on suspicious and positive cases. In order to detect false negative cell studies, surgical pathology reports of uterine malignancies from local hospitals were checked against our alphabetical patients' file to determine whether these women had had any previous cytologic studies performed.

The types of uterine neoplasms detected during the four-year period in which the survey was conducted are shown in Table 1.

The over-all rate of detection during this period was 4.58 cases of uterine cancer per 1,000 women examined, or one case among every 218 women. In addition there were 80 cases with suspicious or positive cell studies in which biopsy confirmation could not be obtained. The rate of detection on the initial examination and on subsequent annual rescreening is shown in Table 2.

When all the cytologic material of the 40 cases of carcinoma *in situ* detected during the first annual rescreening procedure was reviewed, six of them were found to have had suspicious cells on the initial examination. This includes four cases where atypical cells were reported and repeat cellular studies recommended but never obtained. The remaining 34 cases either had "true" false negative examinations on the initial test or developed the lesion in the interim. In only one of these 34 cases, however, had a cervical scraping been submitted.

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Of the two cases in which the *in situ* lesion was detected in the second annual rescreening, neither had suspicious cells the year before.

The marked reduction in the rate of invasive squamous carcinoma seen on the first annual recall examination can be attributed to the proper therapy of the lesions detected on initial examination which are precursors of the invasive stages.

Not only was the number of cases of uterine cancer detected greatly increased after initiation of the project, but the cases were detected in an earlier growth stage. Before the widespread use of cytologic technics, 82 per cent of the cases of cervical cancer detected in the community were in International Stages II or III, whereas in the first year after the project began 86 per cent were in Stages O or I.

In order to compare the accuracy of the vaginal aspirate with the cervical scraping technics, all of the antecedent cytologic material on the confirmed positive cases was reviewed. Each slide was again re-evaluated before and after knowledge of the histologic diagnosis. Any negative cytologic material taken in the six months prior to the time of tissue diagnosis was considered to represent a false negative examination.

In this group of positive cases there were 238 neoplastic lesions detected in the 48,000 women who had one or more sets of vaginal aspirates and cervical scrapings. The comparative evaluation of these technics is shown in Table 3.

TABLE 1. *Number and Types of Uterine Neoplasms Detected by Our Columbus Cancer Survey Project*

| Type of neoplasm | No. of cases | Rate/1,000 women |
|---|---|---|
| Carcinoma *in situ*, cervix | 255 | 2.24 |
| Squamous carcinoma, cervix | 184 | 1.61 |
| Adenocarcinoma, endometrium | 62 | 0.54 |
| Other neoplastic lesions | 22 | 0.19 |
| Total | 523 | 4.58 |

The antecedent cytologic material in these cases included 361 sets of slides. In 30 per cent of these examinations (110 sets of slides) the vaginal aspirate was unsatisfactory, negative, or atypical, whereas the scraping taken at the same time was suspicious or positive. On the other hand, the scraping was negative in only 3 per cent (ten times) when the vaginal aspirate was suspicious. This superiority of the cervical scraping was seen in all types of uterine cancer with the exception of adenocarcinoma of the endometrium, where the two technics were of equal accuracy.

Omitting all cases which at one time or another had produced a suspicious vaginal aspirate, we had 47 cases, 20 per cent of the entire group, which would have been missed if only vaginal aspirates had been

TABLE 2. *The Effect of the Annual Screening Procedure*

| Type of neoplasm | Inital screening (113,758 women) | | 1st annual rescreening (44,009 women) | | 2nd annual rescreening (16,137 women) | |
|---|---|---|---|---|---|---|
| | No. of cases | Rate/1,000 women | No. of cases | Rate/1,000 women | No. of cases | Rate/1,000 women |
| Carcinoma *in situ*, cervix | 213 | 1.87 | 40 | 0.91 | 2 | 0.12 |
| Squamous carcinoma, cervix | 175 | 1.54 | 7 | 0.16 | 2 | 0.12 |
| Adenocarcinoma, endometrium | 49 | 0.43 | 13 | 0.29 | 0 | 0.00 |
| Other neoplastic lesions | 16 | 0.14 | 6 | 0.14 | 0 | 0.00 |
| Total | 453 | 3.98 | 66 | 1.50 | 4 | 0.24 |

TABLE 3. *Comparative Evaluation of the Vaginal Aspirate and Cervical Scraping in 238 Cases of Uterine Malignancy (48,000 Women)*

| Type of neoplasm | No. of cases | No. of VA and CS | VA susp. CS susp. % | VA neg. CS susp. % | VA susp. CS neg. % | VA neg. CS neg. % |
|---|---|---|---|---|---|---|
| Carcinoma *in situ*, cervix | 138 | 234 | 49 | 37 | 3 | 11 |
| Squamous carcinoma, cervix | 64 | 79 | 63 | 23 | 1 | 13 |
| Adenocarcinoma, endometrium | 26 | 35 | 63 | 6 | 8 | 23 |
| Other uterine neoplasms | 10 | 13 | 54 | 23 | 0 | 23 |
| Total | 238 | 361 | 54 | 30 | 3 | 13 |

submitted for evaluation. This includes 35 cases of carcinoma *in situ*, 11 of invasive squamous carcinoma, and one of adenocarcinoma of the endometrium.

## Discussion

From these results it might appear that the use of the vaginal aspirate is not to be recommended because of its decreased efficiency in detecting lesions when compared to the cervical scraping. From a practical standpoint, however, factors other than relative accuracy must also be considered. The simplicity and ease of obtaining a specimen make the vaginal aspirate especially suitable for routine cancer cell testing in doctors' offices and for mass screening projects. Specimens are taken from asymptomatic women on whom the physician might not do a pelvic examination with visualization of the cervix, as is necessary to obtain a scraping. Specimens can even be taken by trained medical aides and nurses. In addition, the vaginal aspirate is valuable for evaluation of hormonal status and detecting inflammatory changes.

Undoubtedly the ideal approach to the detection of individual cases of genital cancer should include a complete history and physical examination with a pelvic examination, vaginal and cervical cytologic studies, and a biopsy of any suspicious areas. This procedure should be encouraged whenever possible, but because of the time and expense involved this does not seem feasible when applied to the screening of large numbers of asymptomatic women. By the more widespread use of simpler routine cytologic technics large numbers of unsuspected malignancies of the female genital tract can be detected.

## Conclusions

Although the cervical scraping was found to be more accurate than the vaginal aspirate in almost all types of genital cancer, the latter is still felt to have its place in routine cancer cell screening of asymptomatic women and in all individual cases when included with a cervical scraping.

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# Physical Properties of Cells in Experimentally Induced Cervical Dysplasia and Cancer*

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IN methylcholanthrene-induced cervical cancer in the mouse we have previously demonstrated a gradually progressive cellular change which can be correlated with the corresponding pathological change in tissue sections.[1] We have also found that once the cytologic atypia progressed to correlate with cervical dysplasia the lesion continued to develop, after a time interval, to invasive carcinoma without further application of a carcinogen.[2]

The aforementioned subjective cytological changes appeared to be gradual, as a matter of fact, so gradual that one is not aware of a change on a day to day basis. It seemed to us then it might be of value to determine by objective criteria the magnitude and rate of change to ascertain if there is a critical level below which the changes are reversible and beyond which they are destined to inevitably progress.

## Materials and Methods

One hundred and fifty virgin female mice of C3H strain, obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, were used in the study. They were maintained on a stock diet with tap water *ad libitim*. Cervical tumorigenesis was induced by 20-methylcholanthrene as a 1 per cent solution in acetone applied directly to the cervix three times a week. A speculum devised by Pratt-Thomas was used to visualize the cervix. Vaginal cells were collected at weekly intervals.

Physiological saline or Ringer's mammalian solution was gently blown into the vagina and then aspirated with a capillary pipette. The aspirate was further diluted with the suspending agent and covered with a No. 1.5 cover slip. The edges were sealed with paraffin to minimize evaporation. Five hundred and twenty-six isolated cells in a relatively clear background, and whose reference beam was not passing through another specimen, were measured. The measurements had to be made within two hours or the cells would tend to imbibe water as evidenced by both increase in area and alterations in the goniometer readings.

The AO Baker Interference Microscope was used with its shearing lens system. The 40× objective and 10× ocular lens were employed for all measurements. The source of illumination was a mercury arc lamp. A ray of light of 0.546 microns in wave length was isolated from this source by means of a reflecting-transmitting filter. The phase change, or optical path difference, was ascertained by using a half-shade eyepiece.[3, 4] The cells which were to be measured were first outlined using a camera lucida, and the areas determined by planimetry. From these data cellular and nuclear areas were computed, relative nuclear/cytoplasmic ratios determined, the anhydrous nuclear mass, and the mass per unit area calculated.

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## Results

The mean cellular area of fresh unfixed cells from normal animals was 796.8 square microns as compared to 350.7 square microns for fixed stained cells from the same animals. The averaged nuclear area for the unfixed cells was 65.7 square microns; for the fixed cells it was 35.2 square microns. The relative nuclear/cytoplasmic ratio for the fresh cells was 9.3 per cent, for the fixed cells 12.3 per cent.

The mean anhydrous nuclear mass of fresh normal cells was $33.4 \times 10^{-12}$ grams, or $0.51 \times 10^{-12}$ grams per square micron. With' further subdivision the mean dry nuclear weight for the parabasal cells was $54.8 \times 10^{-12}$ grams; for the precornified cells $32.1 \times 10^{-12}$ grams; and for the cornified elements $16.8 \times 10^{-12}$ grams.

In basal cell hyperplasia, which occurred following 28 applications of methylcholanthrene, the mean cellular area was 972.7 square microns and the nuclear area was 120.7 square microns, giving a nuclear/cytoplasmic ratio of 18.7 per cent. The averaged anhydrous nuclear mass was $74.8 \times 10^{-12}$ grams, or $0.54 \times 10^{-12}$ grams per square micron. The nuclei of the parabasal cells had an average dry weight of $85.6 \times 10^{-12}$ grams; nuclei of the precornified cells $76.2 \times 10^{-12}$ grams, and nuclei of cornified cells $23.5 \times 10^{-12}$ grams.

From dysplastic lesions the cellular and nuclear areas were 999.1 and 134.0 square microns respectively, giving a nuclear/cytoplasmic ratio of 17.0 per cent. A value of $82.6 \times 10^{-12}$ grams was obtained for the dry nuclear weight, or $0.61 \times 10^{-12}$ grams per square micron.

The mean cellular area for the cells from early invasive cancers was 1,260.2 square microns and that of the nucleus was 198.6 square microns. The nucleus occupied 15.6 per cent of the cell. The anhydrous nuclear mass was $87.4 \times 10^{-12}$ grams, or $0.45 \times 10^{-12}$ grams per square micron.

The nuclei from fully developed cancers had a mean dry mass of $108.5 \times 10^{-12}$ grams, or $0.54 \times 10^{-12}$ grams per square micron. Upon the further division of these abnormal cells into typically cancerous cells and "atypical" cells, a mean anhydrous mass for the cancerous ones was $149.1 \times 10^{-12}$ grams, while for the "atypical" ones it was $75.1 \times 10^{-12}$ grams.

## Summary and Conclusion

In methylcholanthrene treated mice there is a gradually increasing cellular atypia to the stage of invasive cancer.

Objective changes in nuclear mass and cellular area begin to gradually increase after eleven paintings, reaching a peak with invasive carcinoma. The mass per unit area remains essentially unchanged. These objective changes begin to appear considerably earlier than definite subjective cellular morphologic changes.

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# Intranuclear Inclusion Bodies in Smears of Respiratory Secretions

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ACIDOPHILIC inclusion bodies in ciliated cells exfoliated from the human nasal mucosa were first described in 1950 in smears from nasal secretions by William and Marion Bryan,[3, 4] who suggested their probable relationship to viral etiology and recommended cytologic examination of nasal secretions for a differential diagnosis between common cold, allergic reactions and bacterial infections of the upper respiratory tract.

Similar acidophilic inclusion bodies, mostly cytoplasmic and sometimes nuclear, were reported by Papanicolaou in 1956[10] in sputum smears submitted to the Cornell University Medical College Cytology Laboratory for cancer detection. Since the presence of these inclusion bodies in ciliated cells was associated with an extensive degeneration and disintegration of the ciliated components of the bronchial mucosa, Papanicolaou proposed the descriptive term ciliocytophthoria, which literally means destruction of ciliated cells.

It is of interest that, while he observed ciliocytophthoria in frequent association with viral infections, he also noted this phenomenon including the presence of identical acidophilic inclusions in sputa of patients in whom the viral etiology of their pulmonary infections had not been ascertained.

Round acidophilic inclusions, which are seen more often in the cytoplasm than in the nucleus, were also described by Pigarevski, who considered these inclusion bodies to represent a possible nonspecific cellular reaction to the injurious effect of virus.[12] In his opinion, a cytoplasmic formation is produced around virus microcolonies, which are being discarded *in toto* at the time of separation of ciliated tufts, occurring during this process. Apparently, cells without cilia may still regenerate. Pigarevski produced inclusion bodies in the bronchial epithelium of experimental mice infected with influenza virus and found them indistinguishable from those seen in nasal secretions of patients either suffering from influenza or exposed to intranasal spray with live attenuated influenza virus when this method of vaccination was still practiced in the U.S.S.R. Other scientists established that ciliocytophthoria may also result from infection with such other epitheliotropic viruses as adenovirus, varicella, etc.[7, 11] It is currently believed that associated cytoplasmic inclusions do not represent actual congregation of virus particles.[5]

Among several thousand sputum specimens examined annually in the Cytology Laboratory at Hahnemann Medical College in Philadelphia, most are from asymptomatic members of a special study conducted by the American Cancer Society in cooperation with the Veterans Administration.* In smears of these sputa, as well as

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* It is a pilot study aiming at an evaluation of the comparative value of radiologic and cytologic population screening methods in the detection of bronchogenic carcinoma.

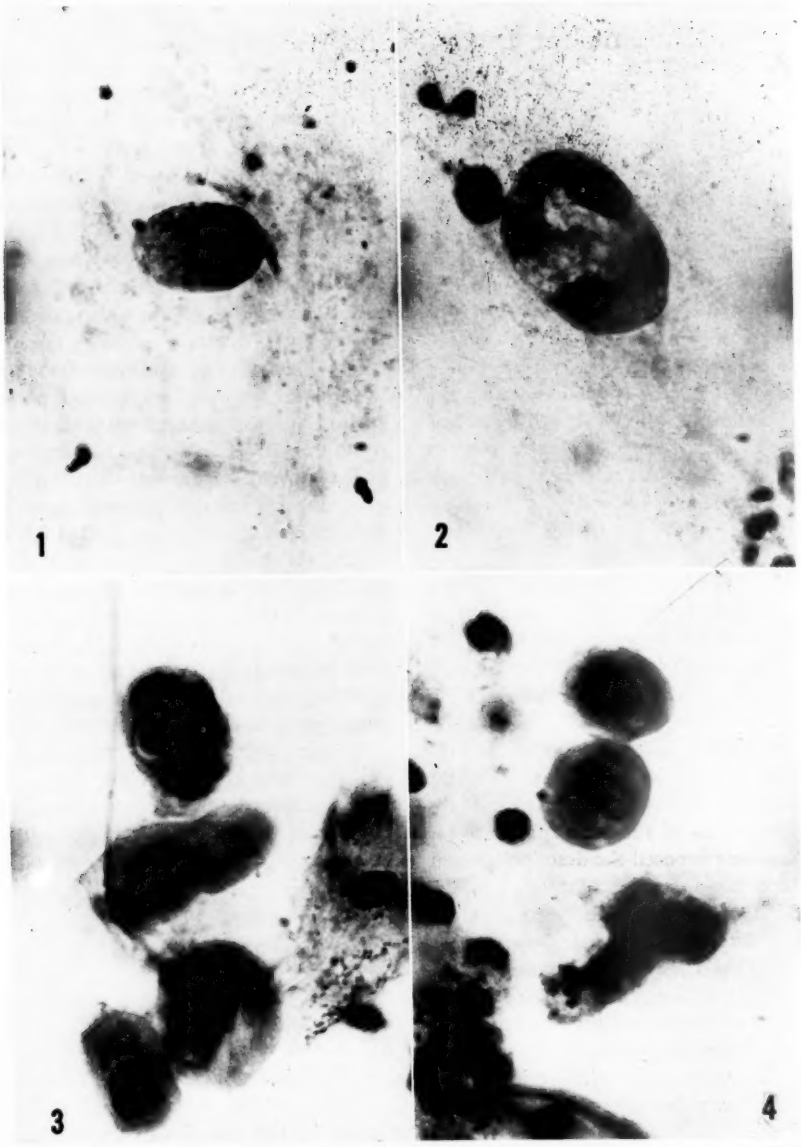


FIG. 1. A degenerated columnar ciliated cell (C.C.P. cell) in sputum, showing large, black, round karyorrhectic fragments and several smaller, round, well outlined, but only slightly deeper stained than the cytoplasm, acidophilic inclusion bodies. FIG. 2. A giant multinucleated C.C.P. cell in which four nuclei are undergoing pyknotic changes, and a single poorly outlined acidophilic inclusion almost of the size of a small nucleus, is present. FIG. 3. A group of "atypical," presumably metaplastic cells in a sputum smear with a sizable single round, acidophilic, cytoplasmic inclusion. FIG. 4. One of two "atypical" parabasal cells in a smear from uterine cervix of a patient with Trichomoniasis, showing an almost identical cytoplasmic inclusion as seen in preceding photograph.

in smears of respiratory secretions of Hahnemann Hospital patients, occasional inclusion bodies associated with ciliocytophthoria were found (Fig. 1, 2). At times, a frequent association of ciliocytophthoria with presence of bizarre cells in smears was quite impressive. This observation may be significant in light of the finding of a somewhat higher incidence of bronchogenic carcinoma among subjects with ciliocytophthoria in Papanicolaou's series.[10]

In addition, a different type of intranuclear inclusion bodies hitherto unreported, to the author's knowledge, in smears of human respiratory secretions, was observed at Hahnemann Cytology Laboratory. These are large, irregular, strictly intranuclear inclusions, amphophilic, but at times acidophilic and/or basophilic, which seem to replace nuclear chromatin and nucleoli by their homogenous bodies, and which are surrounded by vacuoles located on the inner aspect of nuclear membranes. Various modifications of this typical picture were encountered and, in retrospect, presumed to represent different stages of development of these inclusion bodies (Fig. 7, 8, 10, 11-13).

A striking similarity was noted between the appearance of these intranuclear inclusion bodies in smears of sputum and bronchial washings and that of intranuclear inclusion bodies described by several investigators as the result of experimental infection of monkey kidney cells and/or HeLa cells cultures with adenoviruses, Types III, IV and VII[2, 14] (Fig. 17-19).

Although neither the specificity of this second type of inclusion bodies for infection with adenovirus nor the identity of morphologically-indistinguishable formations seen in these different sources of material can be established without adequate serologic, virologic, cytochemical and other specialized technics, it was deemed worthwhile to report and illustrate at the meeting of this Society the nature of our observations.

In order to secure comparable sources of material, we adopted the following procedures:

## Material and Methods

Four flasks with 48-hr. monkey kidney tissue cultures were obtained from Dr. J. E. Prier of the School of Veterinary Medicine, University of Pennsylvania. Cultures in three flasks were infected with Strains III, IV and VII of adenovirus respectively and the culture in the fourth flask served as the non-infected control. Flasks were placed in the incubator at 37 C for 48 hours. Following this, nutrient media were agitated, poured off from each flask and centrifuged separately for 20 minutes at 2,000 rpm. Supernatant fluids were poured off and four smears were prepared from each sediment. Wet smears were immediately placed in ether-alcohol fixative ($\frac{-}{aa}$).

About 2 cc. of isotonic saline was introduced with a pipette into each flask. Monkey kidney cells were then gently scraped off by means of a flattened glass rod, from the site of their growth at the inner wall of the flask, into the saline. The resulting suspension was agitated and used, without centrifugation, for preparation of direct "saline" smears. Again, four smears were prepared from material in each flask and fixed as above.

All smears were stained by a standard Papanicolaou's procedure for staining vaginal smears (EA-series).

Also obtained from Dr. Prier were smears previously prepared from adenovirus-infected monkey kidney cells cultures four-days old, and stained with Wright-Giemsa in his laboratory. In these, he identified typical intranuclear inclusion bodies (Fig. 17-19).

Intranuclear inclusion bodies found in preparations of virus-infected cultures stained in the Cytology Laboratory by Papanicolaou technic (Fig. 14-16) were compared to intranuclear inclusion bodies in smears of human respiratory secretions

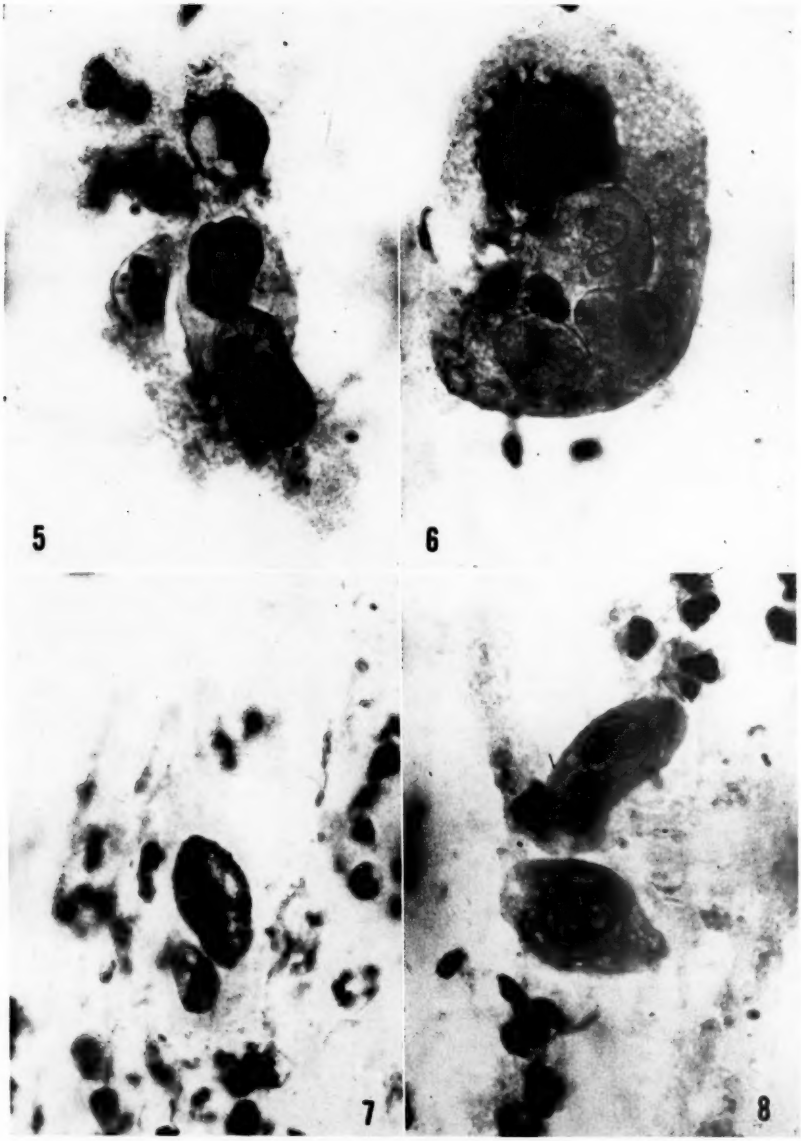


FIG. 5. Several small cytoplasmic inclusions are illustrated within a cluster of "suspicious cells" in a sputum smear. FIG. 6. A giant bizarre cell with several indistinct and confluent nuclei, giving a false impression of a single dark nuclear mass, contains three so called "ghost cells" *i.e.* enucleated keratinized cells appearing as cytoplasmic inclusions. FIG. 7. A large nucleus in the center has a dark inclusion surrounded by lighter peripheral areas. It was observed in sputum smear of a 68-year-old patient with a subsequently discovered very small malignant fungating pharyngeal lesion. FIG. 8. Atypical inclusion bodies appear in a binucleated atypical cell found in a sputum smear. The same smear contained groups of cells with cytoplasmic inclusion bodies and degenerative nuclear changes illustrated in the next photograph.

of several subjects under consideration (Fig. 7, 8, 10, 11-13). Photographs of intranuclear inclusion bodies in all three different types of preparations were prepared under the same magnification and compared to each other and also to various, more familiar, inclusion bodies in smears of respiratory and gynecologic secretions. See Figures 1-6, 9, 20.

## Results

Intranuclear inclusion bodies found in preparations of virus-infected cultures stained in the Cytology Laboratory by the Papanicolaou technic were identical to intranuclear inclusion bodies found in virus-infected cultures stained in Dr. Prier's laboratory with Wright-Giemsa stain and were indistinguishable from intranuclear inclusion bodies found in sputum and bronchial washing smears of several human subjects. Their appearance conformed to the description of adenovirus types: III, IV and VII[2, 14] inclusion bodies observed in monkey kidney tissue cultures and in HeLa cells. The most readily recognizable stage of the development of inclusion bodies observed in smears of respiratory secretions is the one referred to in the literature as a "flower form" and characterized by the presence of a dense intranuclear mass with projections radiating to the nuclear membrane and creating the impression of clear peripheral compartments which sometimes look like vacuoles. No intranuclear bodies were seen in control slides, although in non-infected monkey kidney tissue, a simian virus might be present.[14] The method used in this study was presumably not suitable to determine presence of intranuclear crystals observed by others.[8] Most inclusion bodies were found in direct saline smears.

In one of eight instances in which the above described intranuclear inclusion bodies were observed in smears of respiratory secretions, their presence was associated with findings of intracytoplasmic inclusions, which were not observed in adenovirus-infected tissue culture material (Fig. 9).

Clinical studies of individuals, in whose respiratory secretions intranuclear inclusion bodies covered by this presentation were noted, are beyond the scope of this report.

The following information, however, is available: Of the total of eight subjects whose smears of respiratory secretions revealed intranuclear inclusion bodies, there were six who were non-hospitalized members of the American Cancer Society-Veterans Administration Study Group, and two hospitalized patients from Hahnemann Hospital. Among study members, there were intranuclear inclusion bodies seen in smears of naturally-obtained sputum in four cases and in smears of aerosol-induced expectorates in two cases. Smears with intranuclear inclusion bodies of two hospitalized Hahnemann Hospital patients were prepared from bronchial washings.

Information on Veterans Administration domiciliary subjects was unavailable, in general, because of the rules of the American Cancer Society-Veterans Administration Study. Owing, however, to the courtesy of responsible investigators from Veterans Administration Centers in Dayton, Ohio; Wood, Wisconsin; Biloxi, Mississippi; and Bay Pines, Florida, some fragmentary data were obtained. Five of six subjects are known to be males, presumably elderly, with records of exact age available in three instances, in which it was specified as 59, 61 and 68 years. In the case of the sixth patient, no information was obtained. Among five cases under consideration, one 59-year-old man was known to have repeated episodes of pulmonary infections and to have a fibrotic nodule at the base of the right lung on x-ray, without change in size, approximately one month prior to and two months following collection of sputum. A second subject was known to have a chronic cough and bronchitis, and

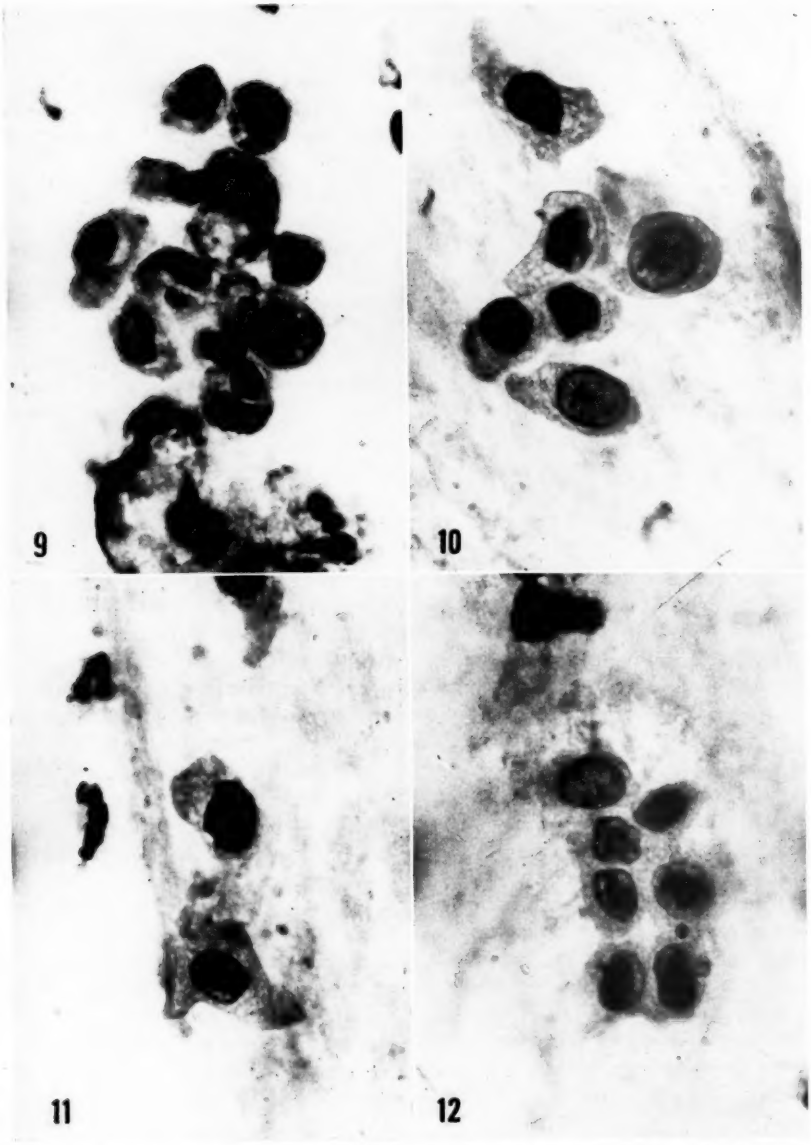


FIG. 9. A group of atypical cells with disintegrating nuclei and cytoplasmic inclusions seen in a sputum smear of the same subject whose nuclear inclusion bodies are shown in the preceding photograph. FIG. 10. Intranuclear inclusion bodies in a sputum smear of a 59-year-old. FIG. 11. Subject with repeated episodes of pulmonary infections. FIG. 12. A group of metaplastic cells with several nuclear inclusion bodies as seen in a sputum smear of a subject with a chronic bronchitis, who developed a severe laryngitis during a flu epidemic a few months after collection of this sample.

a third one apparently had a chronic bronchial asthma. One of these subjects had a severe laryngitis during a flu epidemic several months later and another was found to have a very small malignant fungating pharyngeal lesion. In the case of the two remaining study members, there was no record of any involvement of the respiratory tract. No attempt at the isolation of viral agents from respiratory secretions or to test the blood serum for viral antibodies in the case of these six subjects was done.

Of two hospitalized patients, one was a 40-year-old male, in whom a right lower lobe pneumonitis was clinically diagnosed, prior to finding intranuclear inclusion bodies in bronchial washings; the second patient was a 12-year-old boy with pneumonitis, which did not respond to antibiotic therapy. Throat washings and blood samples of these two subjects were submitted to Dr. Prier for virus isolation and serologic studies respectively. No virus was isolated, however, from either patient.

## Discussion

Findings of unusual intranuclear inclusion bodies in smears of sputum and/or bronchial washings poses several questions; they are as follows: What is the nature of these inclusion bodies? Are they characteristic of infection with adenovirus, with a large group of viruses, or may they appear as a result of non-virus induced nuclear degeneration? Does their presence have any probable relationship to pathogenesis of cancer?

Although these questions cannot be answered at the present time, some light may be thrown on the nature of pertinent speculations.

Inclusion bodies are structures enclosed within the nucleus or the cytoplasm and the presence of which is commonly, but not necessarily, associated with a viral infection. In a recent review of the relation of viruses to inclusion bodies, Pinkerton brings up the history of the description of the molluscum body in 1841 and the Bollinger body of fowlpox in 1873, which were long believed to be protozoa or fungi. A later concept that certain nuclear and cytoplasmic inclusion bodies might be colonies of viral particles was confirmed in the case of several of the larger viruses by direct observation. The nature of the inclusion bodies associated with smaller viruses has recently been partially clarified by the use of the electron microscope. It should be emphasized that new virological concepts are being developed concerning host-parasite relationship and the method of viral multiplication. Thus, small viruses are believed to multiply by the breakdown of virus particles into sub-units, which are reduplicated separately by diversion of host enzymes and which recombine later to form mature infective particles. The multiplication of these sub-units may occur in different parts of the cell, such as nucleus and cytoplasm.[13] We may deduce that in the case of viral infection associated with nuclear and cytoplasmic inclusion bodies, it is probable that inclusions in different parts of the cell may represent an aggregation of virus bodies in incomplete or complete form of development. That appearance of nuclear inclusions precedes the appearance of cytoplasmic inclusions was shown in a study concerned with the salivary gland virus infection.[9] In the same study, McMillan refers to the existence of inclusion bodies induced chemically by bismuth and aluminum hydroxide. One can speculate further that these chemicals stimulate intracellular multiplication of virus in the presence of a latent infection. Such a possibility may also be considered in the course of chemical carcinogenesis. Experiments of the late Francisco-Duran Reynals certainly point in this direction.[6]

It may be of interest to mention that upon a recent review of a false positive cytologic report of a gynecologic case from

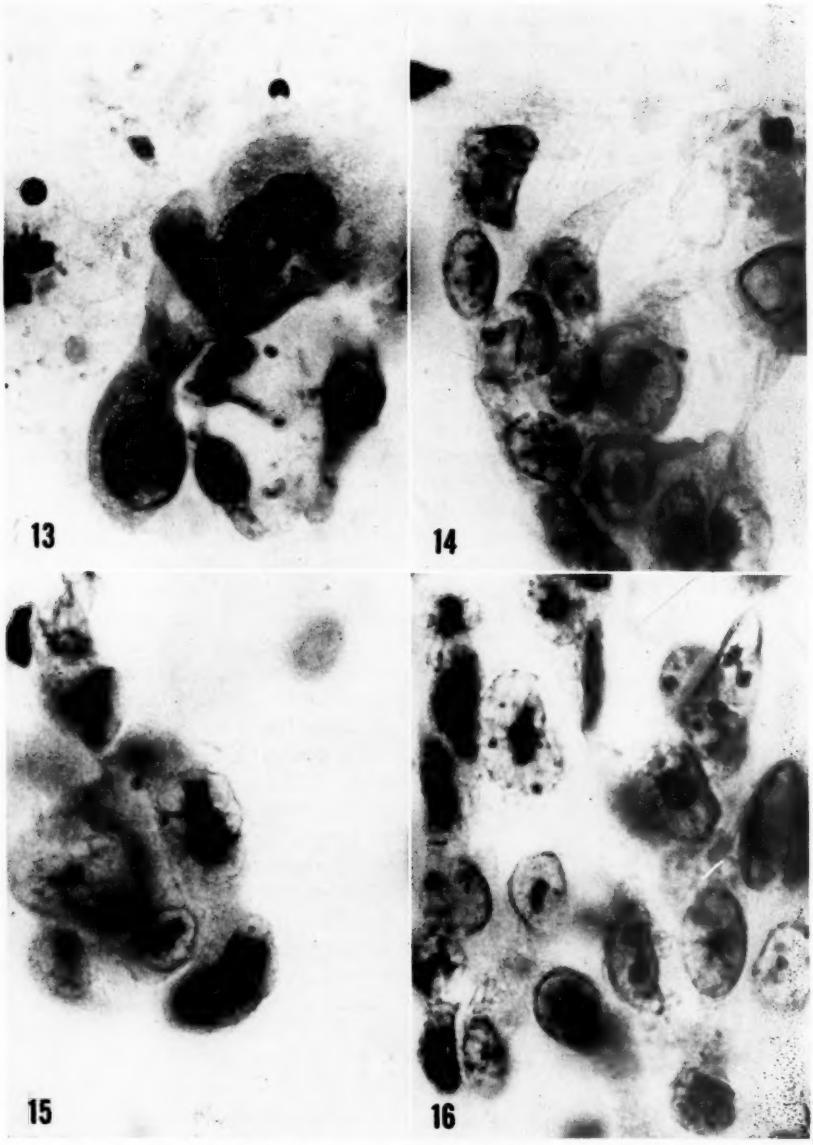


FIG. 13. Nuclear inclusion bodies in a bronchial washing of an adolescent with atypical Pneumonitis. FIG. 14. Examples of adeno-virus nuclear inclusion bodies in monkey kidney cells, Adenovirus type III. Stained with Papanicolaou stain. FIG. 15. Examples of adeno-virus nuclear inclusion bodies in monkey kidney cells, Adenovirus type IV. Stained with Papanicolaou stain. FIG. 16. Examples of adeno-virus nuclear inclusion bodies in monkey kidney cells, Adenovirus type VII. Stained with Papanicolaou stain.

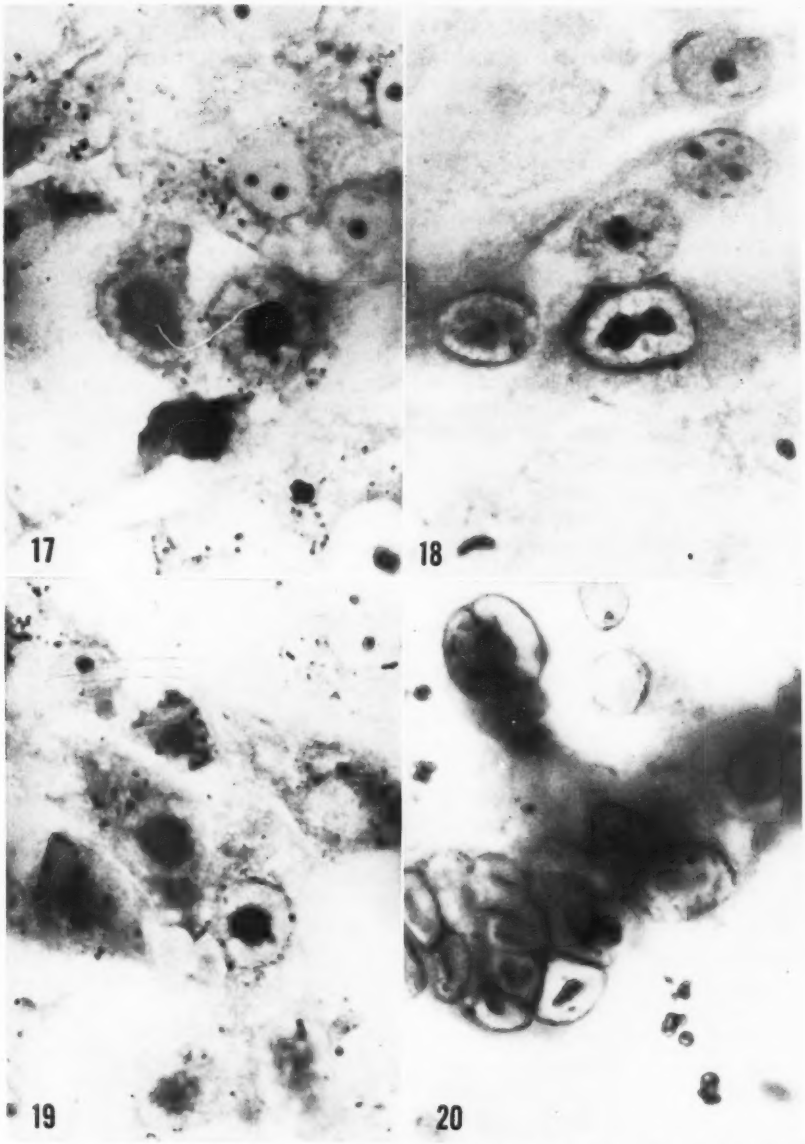


FIG. 17. Examples of adeno-virus nuclear inclusion bodies in monkey kidney cells, Adenovirus type III. Stained with Wright-Giemsa stain. FIG. 18. Examples of adeno-virus nuclear inclusion bodies in monkey kidney cells, Adenovirus type IV. Stained with Wright-Giemsa stain. FIG. 19. Examples of adeno-virus nuclear inclusion bodies in monkey kidney cells, Adenovirus type VII. Stained with Wright-Giemsa stain. FIG. 20. Intranuclear inclusion bodies in a cluster of abnormal cells in an endocervical smear of a patient who had vaginal burns produced by douching with a concentrated solution of Potassium Hypermanganate.

the Hahnemann Cytology Laboratory, intranuclear inclusion bodies were seen in cells forming a crucial cluster responsible for the suspicion of a malignant neoplasm. The patient in question had burns in the vaginal vault, produced by a concentrated solution of Potassium Permanganate (Fig. 20).

Intranuclear inclusion bodies serving as a basis for this report, although indistinguishable in stained preparations studied through light microscope from those produced by adenoviruses types III, IV and VII, in HeLa cells and monkey kidney tissue and identified as viral DNA[1] cannot be attributed to adenovirus infection without proper virus isolation and immunologic studies. In some cases, cytologic findings may be the first ones to alert clinicians to the need for such additional studies from the standpoint of diagnosis of different etiologic agents involved in the group of viral pneumonitis. From the standpoint of a student of etiologic factors in pathogenesis of bronchogenic carcinoma, the association of certain cellular features suspicious of a malignant neoplasm with viral infection is of obvious interest.

While the field of Exfoliative Cytology, until recently, has been singularly free from worries about the nature and specificity of the inclusion bodies and a possible role of viruses in pathogenesis of cancer, this is apparently no longer the case. A day may come when Exfoliative Cytology will not only invade, but gain a firm foothold in the field of diagnosis of infectious diseases, whenever infectious agents produce morphologic changes detectable through light microscope in fixed and stained preparations of desquamated cells. Studies on correlation of morphologic observations and clinical findings should be greatly encouraged.


## Summary

Unusual intranuclear inclusion bodies were observed in sputum and bronchial washings of several individuals. The appearance of these structures in smears prepared by Papanicolaou technic was indistinguishable from the appearance of inclusion bodies induced by adenoviruses in monkey kidney cells. Smears prepared from virus infected tissue cultures were stained, for comparison, by Papanicolaou as well as Wright-Giemsa stain.

In the presence of intranuclear inclusion bodies, cytologic examination of respiratory secretions may be of special value to the clinicians in alerting them to the need for pertinent virus isolation and immunologic studies.


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